Glia-dependent TGF-β signaling, acting independently of the TH17 pathway, is critical for initiation of murine autoimmune encephalomyelitis

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Autoimmune encephalomyelitis, a mouse model for multiple sclerosis, is characterized by the activation of immune cells, demyelination of axons in the CNS, and paralysis. We found that TGF-β1 synthesis in glial cells and TGF-β–induced signaling in the CNS were activated several days before the onset of paralysis in mice with autoimmune encephalomyelitis. While early production of TGF-β1 was observed in glial cells TGF-β signaling was activated in neurons and later in infiltrating T cells in inflammatory lesions. Systemic treatment with a pharmacological inhibitor of TGF-β signaling ameliorated the paralytic disease and reduced the accumulation of pathogenic T cells and expression of IL-6 in the CNS. Priming of peripheral T cells was not altered, nor was the generation of TH17 cells, indicating that this effect was directed within the brain, yet affected the immune system. These results suggest that early production of TGF-β1 in the CNS creates a permissive and dangerous environment for the initiation of autoimmune inflammation, providing a rare example of the brain modulating the immune system. Importantly, inhibition of TGF-β signaling may have benefits in the treatment of the acute phase of autoimmune CNS inflammation.

Introduction

MS is an inflammatory demyelinating disease of the CNS. It is characterized by myelin destruction and axonal loss leading to progressive disability (1, 2). EAE is the prototypical animal model for MS and can be induced in susceptible rodents by immunization with myelin antigens such as myelin oligodendrocyte glycoprotein (MOG) or by adoptive transfer of myelin-specific Th1 CD4+ T cells (3, 4). As a result, inflammatory cells consisting mostly of CD4+ T cells, monocytes, and macrophages infiltrate the CNS, leading to demyelination and neuronal damage (3, 4). While a great deal is known about the development of autoimmune T cells in the periphery from experimental animal models, it is still unclear how these autoreactive T cells take residence in the CNS, a milieu classically considered as “out of bounds” or “immune privileged,” and how they then exert their destructive properties.

TGF-βs are members of a superfamily of multifunctional cytokines with key functions in development, patterning, carcinogenesis, fibrosis, and immune responses (5, 6). In the immune system, TGF-β1 is involved in the induction of immune tolerance by regulating T cell proliferation, differentiation, and survival (7, 8). In addition, TGF-β1 regulates the initiation and resolution of inflammatory responses through its effects on chemotaxis, activation, and survival of immune cells (7, 8). Among the 3 TGF-β isoforms (TGF-β1, -β2, and -β3), TGF-β1 is the predominant isoform expressed in the immune system (9). In the CNS, TGF-β1 helps orchestrate the response to brain injury and has been implicated in a number of disorders, including stroke, Parkinson disease, and Alzheimer disease (10). TGF-β1 and its receptors are expressed in the CNS inflammatory lesions of MS patients and in various animal models of the disease (11–13), suggesting a role in the disease process. However, their role in EAE seems paradoxical. Systemic administration of exogenous TGF-β1 in mice prevents or inhibits EAE (13, 14), and administration of TGF-β–neutralizing antibodies enhances disease severity (15), suggesting a protective role of TGF-β1 in EAE. In contrast, overproduction of TGF-β1 locally in the brain results in more severe EAE, with earlier disease onset and larger mononuclear cell infiltrates in the CNS (16). This discrepancy between the roles of TGF-β suggests that they may have autonomous functions. Little consideration has been given to possible unidirectional influences of the brain on the immune system.

The recent identification of TGF-β1 as a crucial factor for the differentiation of TH17 (17–19), an IL-17–producing T cell subset believed to be autoreactive and disease promoting (20), further supports a pathogenic role of TGF-β1 in EAE (21). While these recent studies highlight the critical role of TGF-β1 in the pathogenesis of EAE and MS in the priming and expansion of autoreactive T cells, they do not approach the important question of how TGF-β1, produced in the CNS, modulates the disease process. We report here that TGF-β1 plays a role that temporally occurs first

Nonstandard abbreviations used: ALK5, activin-like kinase receptor 5; dpi, day(s) post immunization; GFAP, glial fibrillary acidic protein; MOG, myelin oligodendrocyte glycoprotein; PT, pertussis toxin; RFP, red fluorescent protein; SBE, smad-bind element(s).

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in the inflammatory process in the CNS and is distinct from its role outside the brain on T cells of the immune system. This is an example of the brain signaling the immune system, a rare situation with at least some precedents (22–24). Examples of bidirectional interactions between brain and the immune system occur in the febrile response while examples of the immune system influencing the brain are at the heart of our understanding of autoimmune diseases such as EAE (23, 25).

Results

Astrogliosis and neuroinflammation precede clinical signs in EAE. To understand the early events in the pathogenesis of autoimmune disease in the brain, we studied the activation of astrocytes during EAE in a transgenic reporter mouse in vivo (26). Astrocytes are rapidly activated at sites of inflammation, as demonstrated by increased expression of glial fibrillary acidic protein (GFAP) (27, 28). Astrocyte activation can be monitored noninvasively in vivo using bioluminescence imaging in mice expressing luciferase under the control of a GFAP promoter (26). Induction of EAE in these mice by immunization with MOG\textsubscript{35–55} peptide and adjuvants led to a small but detectable increase in bioluminescence in the brain as early as 3 days post immunization (dpi) (data not shown) and to a consistently significant increase at 7 dpi (Figure 1A). Importantly, mice did not show any clinical signs of disease until 11 dpi (Figure 1B), consistent with astrocyte activation preceding clinical manifestations by several days (27, 28). Similar results were also obtained in the spinal cord (Figure 1, A and B).

In agreement with these imaging observations, immunohistochemical analysis of brain sections from mice sacrificed at various time points after immunization showed early activation of astrocytes (GFAP) and microglia (CD68) several days before clinical onset of disease (Figure 1C). Glial activation (gliosis) was detected as early as 3 dpi in the cerebellum and was less prominent in brain stem and spinal cord (data not shown). Interestingly, significant T cell infiltrates were not seen until 10 dpi, just before clinical symptoms were noticeable (Figure 1C). Thus, local CNS...
responses precede significant T cell infiltration and clinical disease onset, underlining the importance of the local CNS environment in the development of the disease.

**Early upregulation of TGF-β1 and activation of TGF-β signaling** in the CNS. TGF-β1 is an important injury-response factor and modulator of immune responses (7) and is produced locally in the brain during MS and EAE (11–13). Indeed, strong TGF-β1 immunoreactivity was observed in the CNS of mice immunized with MOG35-55 peptide as early as 7 dpi (Figure 2, B and C), consistent with the early timing of activation of glial cells. TGF-β1 immunoreactivity was present at 7 and 14 dpi in cerebellum (Figure 2), cortex, brain stem, and spinal cord (data not shown), which are brain regions highly susceptible to T cell infiltration and demyelination. Double immunolabeling and confocal microscopy identified microglia (Figure 2D) and astrocytes (Figure 2E) as the main TGF-β1-producing cells, consistent with previous reports in acute brain injury (29, 30). CD4+ T cells showed no significant TGF-β1 immunoreactivity at 7 (Figure 2F) or 14 dpi (not shown).

To determine whether increased TGF-β1 production leads to activation of TGF-β signaling, we used SBE-luc (SBE, smad-bind- ing element) mice, which harbor a TGF-β-responsive luciferase reporter gene and have been shown to reliably indicate activation of TGF-β signaling (31, 32). Thus, TGF-β signaling in these animals can be monitored in vivo over time by bioluminescence imaging. Immunization of SBE-luc mice with MOG35-55 peptide resulted in bioluminescence emitting first from the brain and later from the spinal cord, indicating early activation of TGF-β signaling (Figure 3A). Consistent with GFAP-luc mice, this signal appeared at least 4 days before clinical signs were apparent (Figure 3A), indicating a role of TGF-β signaling in the earliest phase of EAE. Bioluminescence measurements were confirmed by tissue luciferase activity assay in both brain and spinal cord (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI31763DS1).

To identify the cellular source of the bioluminescence signal and because no specific antibodies are available to detect luciferase expression in brain tissue (31, 32), we generated new reporter mice for the TGF-β signaling pathway. These SBE-lucRT mice express a trifusion protein containing luciferase, red fluorescent protein (RFP), and thymidine kinase (33) under the control of the same SBE promoter as the original SBE-luc reporter mice (31, 32). In agreement with SBE-luc mice (32), baseline TGF-β signaling in SBE-lucRT mice was detected in large pyramidal neurons in the hippocampus and cortex (data not shown) and Purkinje cells of the cerebellum (Figure 3B). Immunization with MOG35-55 led to infiltration of T cells and increased RFP immunoreactivity starting at 7 dpi in neurons (Figure 3, C and D), consistent with bioluminescence imaging. Importantly, infiltrated CD4+ T cells were RFP immunoreactive as well (Figure 3, E and H), indicating that TGF-β signaling is activated in these cells. Microglia (Figure 3F) and astrocytes (Figure 3G) showed no significant RFP immunoreactivity. Similar results were obtained from immunostaining of phosphorylated Smad2 (32), a primary downstream target of the TGF-β signaling pathway (data not shown). These results demonstrate that TGF-β signaling is activated early after MOG35-55 immunization and increases with disease progression and that neurons and later CD4+ T cells are the main targets of TGF-β signaling in the brain at this stage of the disease.

Induction of EAE requires antigen (MOG35-55) to be emulsified in CFA, an immune stimulant that recruits innate immune responses in the periphery and possibly in the CNS (3). In addition, pertussis toxin (PT) is administered to open the blood-brain barrier and provide entry of T cells and macrophages into the CNS (34). To determine whether adjuvant or MOG35-55 peptide might cause the early increase in TGF-β1 production or the activation of TGF-β signaling, we injected SBE-lucRT mice with CFA alone, PT alone, or combinations thereof with MOG35-55 and analyzed their brains. Mice receiving CFA alone, but not those receiving PT alone, showed a significant increase in TGF-β1 immunoreactiv-
ity in the CNS, similar to mice receiving the full immunization cocktail (MOG<sub>35-55</sub> emulsified in CFA plus PT; Supplemental Figure 2). Combining MOG<sub>35-55</sub> or PT with CFA did not further increase TGF-β<sub>1</sub> immunoreactivity. Similar results were obtained from biochemical measurements of tissue luciferase activity (data not shown), demonstrating that CFA alone can activate TGF-β<sub>1</sub> signaling in the CNS. Early studies showed that CFA preimmunization protects against subsequent EAE induction (35). It would be interesting to investigate whether this protection is due to increased TGF-β<sub>1</sub>.

Astrocyte-targeted overexpression of TGF-β<sub>1</sub> results in earlier onset of EAE. These results raise the question of whether the early activation of TGF-β<sub>1</sub> signaling delays or promotes disease. In support of a proinflammatory role for TGF-β<sub>1</sub> in EAE, transgenic mice overproducing TGF-β<sub>1</sub> in astrocytes (GFAP-pTGF-β<sub>1</sub>) (36) showed earlier disease onset and more severe disease when immunized with spinal cord homogenate in CFA (16). In concordance with these results, we show here that the same TGF-β<sub>1</sub> transgenic mice backcrossed onto a C57BL/6 genetic background and immunized with MOG<sub>35-55</sub> in CFA also had significantly earlier disease onset and more severe disease than littermate controls (Supplemental Table 1). Thus, TGF-β<sub>1</sub> produced locally in the brain exacerbated EAE. To determine whether this effect of TGF-β<sub>1</sub> is due to altered priming of encephalitogenic T cell responses, we isolated T cells from the spleen and tested their in vitro recall responses to secondary exposure to MOG<sub>35-55</sub>. T cells derived from TGF-β<sub>1</sub> transgenic mice showed no significant difference in recall responses to MOG<sub>35-55</sub> compared with those of nontransgenic mice (Supplemental Figure 3). In addition, astrocyte-derived TGF-β<sub>1</sub> in these mice did not significantly change the production of IL-6, IL-12, IL-17, and IL-23 in cultured splenocytes (Supplemental Figure 3).

### Table 1
Pharmacological inhibition of TGF-β signaling ameliorates EAE

<table>
<thead>
<tr>
<th>Group</th>
<th>Day of onset (range)</th>
<th>Peak clinical (spinal cord)</th>
<th>Peak body weight loss (% of preinjury)</th>
<th>Peak brain BLI (fold induction)</th>
<th>Peak spinal cord BLI (fold induction)</th>
<th>T cell infiltration (spinal cord)</th>
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<tr>
<td>SBE-luc mice</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>EAE + PBS</td>
<td>9.3 ± 0.2 (9–11)</td>
<td>3.42 ± 0.5 (d14)</td>
<td>86.4 ± 0.04</td>
<td>9.8 ± 2.5</td>
<td>5.7 ± 0.5</td>
<td>127.88 ± 26.87</td>
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<tr>
<td>EAE + IN-1130</td>
<td>13.2 ± 0.1 (13–14)&lt;sup&gt;A&lt;/sup&gt;</td>
<td>1.33 ± 0.5&lt;sup&gt;A&lt;/sup&gt;</td>
<td>94.9 ± 0.04</td>
<td>4.8 ± 1.4&lt;sup&gt;B&lt;/sup&gt;</td>
<td>3.8 ± 0.4&lt;sup&gt;B&lt;/sup&gt;</td>
<td>4.67 ± 1.67&lt;sup&gt;B&lt;/sup&gt; (d11)</td>
</tr>
<tr>
<td>GFAP-luc mice</td>
<td></td>
<td></td>
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<tr>
<td>EAE + PBS</td>
<td>10.5 ± 0.3 (10–11)</td>
<td>4.25 ± 0.3</td>
<td>81.4 ± 0.02</td>
<td>95.0 ± 15.5</td>
<td>57.5 ± 14.1</td>
<td>466.80 ± 47.90</td>
</tr>
<tr>
<td>EAE + IN-1130</td>
<td>11.3 ± 0.8 (10–13)</td>
<td>2.75 ± 0.9&lt;sup&gt;A&lt;/sup&gt;</td>
<td>91.9 ± 0.04&lt;sup&gt;B&lt;/sup&gt;</td>
<td>28.3 ± 7.5&lt;sup&gt;B&lt;/sup&gt;</td>
<td>25.3 ± 12.1&lt;sup&gt;B&lt;/sup&gt;</td>
<td>230.6 ± 42.28&lt;sup&gt;B&lt;/sup&gt; (d14)</td>
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Inhibition of TGF-β signaling by IN-1130 ameliorates EAE. Data are expressed as mean ± SEM. <sup>A</sup><sub>P < 0.01</sub>, <sup>B</sup><sub>P < 0.05</sub> compared with EAE + PBS by unpaired Student’s t test. BLI, bioluminescence imaging signals.
Pharmacological inhibition of TGF-β signaling ameliorates EAE. GFAP-luc mice (A–C, n = 4–7 per group) or SBE-luc mice (D, n = 10–12 per group) were immunized with MOG$_{35-55}$ emulsified in CFA and treated with the TGF-β receptor kinase inhibitor IN-1130 (closed symbols) or PBS (open symbols) from 1–14 (A–C) or 3–14 (D) dpi. Daily bioluminescence and clinical assessment (weight loss, C; clinical score, D) were recorded in a blinded manner. Bioluminescence is shown from representative mice of each group individually (A) or as mean ± SEM (B). Similar results were obtained in 2 independent experiments. Data are shown as mean ± SEM.

Table 1. Similar to TGF-β1 transgenic mice, IN-1130–treated mice showed no significant changes in peripheral T cell priming (Supplemental Figure 5).

Inhibition of TGF-β signaling leads to reduced T cell infiltration and TGF-β1 expression in the CNS. Given the observation that MOG$_{35-55}$–immunized mice treated with IN-1130 had no overt changes in their peripheral immune response, we tested to determine whether IN-1130 affects the accumulation of pathogenic effector cells in the CNS. Indeed, treatment with IN-1130 dramatically reduced the number of CD3+ T cells in the spinal cord at 11 dpi (SBE-luc mice) (Figure 5, A and B) and 14 dpi (GFAP-luc mice) (Figure 5C and Table 1). To further confirm the inhibition of TGF-β signaling by IN-1130, we used quantitative real-time PCR analysis to determine the expression of TGF-β1. TGF-β1 was upregulated in the CNS at 14 dpi, but its induction was significantly reduced in IN-1130–treated mice (Figure 5D). TGF-β1 can induce its own synthesis (38), and previous findings showed that IN-1130 treatment reduced TGF-β1 gene expression in an animal model of kidney fibrosis (37). Moreover, IN-1130 treatment almost completely inhibited the upregulation of IL-6 in the CNS (Figure 5E), a proinflammatory cytokine induced by TGF-β1 and critical in the induction of a number of experimental autoimmune conditions including EAE (39). Interestingly, the expression of IL-23, a cytokine important in Th17 expansion (40), was not significantly affected by IN-1130 treatment (Figure 5F). In agreement with a limited role of IL-23 in the TGF-β1–dependent effects reported here, treatment of EAE with anti–IL-23 antibodies in SBE-lucRT mice did not significantly reduce TGF-β signaling despite its protective effect on the clinical progression of disease (Supplemental Figure 6). Together, our findings show that reduced TGF-β signaling in the CNS results in the accumulation of fewer effector T cells and thus less CNS inflammation and disease.

Inhibition of TGF-β signaling does not significantly change the percentage of CD4+ T cell subsets in brain or spleen. To determine whether reduced accumulation of effector T cells in the brains of IN-1130–treated mice is due to the reduction of a specific T cell subset, we isolated lymphocytes from spinal cords and spleens of mice at 20 dpi and measured total CD4+ infiltration (CD4+ cells) and CD4+ T cell subsets based on expression of IL-4, IFN-γ, Foxp3, or IL-17. IN-1130 treatment did not change the overall number of CD4+ T cells in the spleen (Figure 6A) or the percentage of the various T cell subsets (Figure 6C), consistent with the finding that IN-1130 does not change the production of IL-6, IL-12, IL-17, and IL-23 in splenocytes (Supplemental Figure 5). In contrast, IN-1130 treatment strongly reduced the number of CD4+ T cells in the spinal cord (Figure 6A). Again, the percentage of CD4+ T cell subsets between the 2 groups was not statistically significant (Figure 6B).
To determine in which T cells TGF-β signaling is activated in EAE, we immunized SBE-lucRT mice and measured the percentage of RFP-expressing (TGF-β signaling activated) CD4+ T cell subsets in the spinal cord and spleen. Immunohistochemistry showed activated TGF-β signaling in infiltrated T cells in the CNS (Figure 3), and foci of similar T cells were found in the spleen (Figure 7, A and B). In mice with EAE, about 30% of CD4+ cells in the spleen and 50% of CD4+ cells in the spinal cord were RFP positive. Consistent with the bioluminescence imaging in SBE-luc mice, IN-1130 treatment led to significantly reduced RFP reporter gene expression in CD4+ T cells isolated from both spinal cord and spleen in SBE-lucRT mice (Figure 7C), indicating inhibition of TGF-β signaling by IN-1130. However, the composition of the RFP+ T cells in IN-1130–treated mice was not significantly different from that in PBS-treated mice in both spinal cord (Figure 7, D and E) and spleen (Figure 7, D and F), further supporting that inhibiting TGF-β signaling by IN-1130 does not exert T cell subset–specific effects. The analysis of T cell subsets shows that reduced CD4+ T cell accumulation in the brain after IN-1130 treatment is not due to a specific reduction of a major T cell subset.

Discussion

The present study demonstrates that the local environment in the CNS is critical in the initiation of autoimmune encephalitis. Using luciferase reporter mice and in vivo bioluminescence imaging, we discovered that astrocytes, microglia, and Smad-dependent TGF-β signaling are activated early in the CNS, several days before clinical onset of disease. The adjuvant CFA is likely sufficient to initiate these early events, leading to increased production of TGF-β1 in astrocytes and microglia. Consequently, TGF-β signaling is activated in neurons and later in infiltrating T cells. The inhibition of TGF-β signaling delayed and ameliorated progression of autoimmune encephalomyelitis by significantly reducing the accumulation of T cells in the CNS but not by altering the priming of peripheral autoreactive T cells or the expansion of specific CD4+ T cell subsets.

In autoimmune inflammatory disease, autoreactive T cells typically generated in lymphoid organs not only need to infiltrate but also need to successfully accumulate in the target organ to cause disease. TGF-β1 can participate in all these processes by regulating T cell proliferation, differentiation, and apoptosis as well as chemotaxis and homing (7, 8). These site-specific actions may explain the seemingly paradoxical effects of TGF-β1 in inhibiting or promoting inflammatory responses and autoimmunity. Indeed in EAE, TGF-β1 was initially discovered to be immunosuppressive and to inhibit disease (13, 14) but is now recognized as a critical factor in the genesis of autoreactive, IL-17–producing T cells (17–19). The current study demonstrates that TGF-β1 in the CNS may be critical in promoting disease. We propose that CNS-produced TGF-β1 creates a permissive environment for the accumulation of pathogenic T cells and propagation of an autoimmune response.
Our findings parallel observations in an experimental model of arthritis, where administering TGF-β1 locally in the joint exacerbates the inflammatory response and aggravates disease (41), but systemic inoculation inhibits inflammation (42). Similarly, blocking endogenous TGF-β1 by systemic injection of anti-TGF-β1 neutralizing antibody exacerbates arthritis (43), but local blockade of TGF-β1 ameliorates ongoing inflammation (44).

Pharmacological inhibition of TGF-β signaling with the ALK5 kinase inhibitor IN-1130 resulted in delayed disease onset and overall less disease in MOG<sub>35-55</sub>-immunized mice (Figure 4 and Table 1). It was shown recently that transgenic mice with CD4<sup>+</sup> T cells expressing a dominant-negative form of the TGF-β type II receptor and thus, not responsive to TGF-β1, failed to generate TH17 cells and did not develop EAE (20). Interestingly, inhibition of TGF-β signaling with IN-1130 did not alter priming of MOG<sub>35-55</sub>-specific T cells nor their production of IL-17 or other cytokines involved in the generation of TH17 cells (Supplemental Figure 5). This suggests IN-1130 was not sufficient to interfere with TGF-β signaling at the dendritic cell–T cell interface (20) and to block the generation of autoreactive TH17 cells in the periphery. IN-1130 also did not change the percentage of CD4<sup>+</sup> T cell subsets in the CNS or the periphery (Figures 6 and 7). Nevertheless, IN-1130 treatment strongly inhibited GFAP- and SBE-dependent reporter gene transcription (Figure 4), reduced TGF-β1 and IL-6 mRNA levels in the CNS (Figure 5), reduced the percentage of CD4<sup>+</sup> cells with activated TGF-β signaling in spinal cord and spleen (Figure 7), and most importantly, potently suppressed the accumulation of T cells in the CNS (Figure 5 and Table 1). These results strongly indicate that the TGF-β–dependent effects observed after IN-1130 treatment go beyond the generation of TH17 cells and involve what we believe are novel immune-modulatory effects of TGF-β1 in the CNS. This is also an example of the modulation of the immune system by the brain (23). Neurons have a crucial role in the regulation of the T cell response. Function of Tregs has recently been shown to be modulated by neuronal signals (22), and interestingly, IL-6 is a potent inhibitor of Treg differentiation (17). Finally, the modulation of weight loss (Figure 4 and Table 1) may indicate an interaction between the immune system and the hypothalamic pituitary axis, which is critical in leptin regulation (45). Leptin is both a fat modulator and a Th1 cytokine (proinflammatory).

Further emphasizing the importance of TGF-β1 in the CNS during EAE, the overproduction of a constitutively active form of TGF-β1 in astrocytes of transgenic mice resulted in an acceleration and exacerbation of disease after immunization with spinal cord homogenate (16) or MOG<sub>35-55</sub> peptide (Supplemental Table 1). Again, T cell priming and production of cytokines after recall stimulation with MOG<sub>35-55</sub> in culture were not affected by this tissue-restricted overexpression of TGF-β1 (Supplemental Figure 3). In a related experiment, astrocyte-restricted overexpression of TGF-β1 in a mouse model for Alzheimer disease resulted in increased T cell accumulation in the brain after immunization with the peptide that accumulates in the brains of these mice (46), indicating that the effect of TGF-β1 on cerebral accumulation of T cells is not restricted to myelin-specific cells. Because infiltrating T cells in EAE brains showed prominent activation of TGF-β1 signaling (Figures 3 and 7), it is tempting to speculate that at least some of the disease-attenuating effect observed in mice with TGF-β1 signaling–deficient T cells (20) is related to their inability to respond to TGF-β1 in the CNS.

How TGF-β1 regulates T cell function and facilitates accumulation in the CNS requires further study. TGF-β1 is strongly chemotactic for monocytes (47), neutrophils (48), and T cells (49) and induces the production of chemokines such as monocyte chemoattractant protein 1 (MCP-1) and several chemokine receptors (8). Besides this effect on chemotaxis, TGF-β1 might increase homing to the brain by increasing adhesion molecules on infiltrating cells and the vasculature (8). Interestingly, unmanipulated GFAP-pTGF-β1 mice, which produce significant amounts of bioactive TGF-β1 in their brains, only slowly accumulate T cells in the brain with aging (46). This suggests that, at least in the absence of autoimmune T cells, TGF-β1 does not significantly increase chemotaxis or homing of T cells. Instead, TGF-β1 may increase the survival of infiltrating T cells via activation of antiapoptotic and prosurvival pathways (7). In the context of an immune reaction, such a function would obviously be ill-fated.

CFA is required to induce EAE, and we show that it is sufficient to activate TGF-β1 production from glial cells and TGF-β1 signaling in the CNS (Supplemental Figure 2). The effect of CFA is likely due to the presence of Mycobacterium tuberculosis, which strongly activates monocytes to produce TGF-β1 in cell culture (50) and can also
convert latent TGF-β1 to its bioactive form (51). Accordingly, these bacteria may be responsible for the observed increase in TGF-β1 production in microglia and astrocytes in our model (Figure 2). M. tuberculosis can also stimulate IL-17 production in T cells (52) and induce TH17 cells in a TGF-β1–dependent fashion (20). Given the observation that LPS is also a strong inducer of TGF-β signaling in the CNS (31) and has been shown to promote relapses in EAE (53), it is tempting to speculate that bacterial infections may similarly promote relapses in MS patients (1) by creating a more permissive, proinflammatory environment in the CNS. Activation of glial cells by microbial signals is mediated primarily through pattern recognition receptors, of which TLRs are key participants (54–56). TLRs recognize microbial structural motifs referred to as pathogen-associated molecular patterns (PAMP). One TLR molecule, TLR4, binds LPS from Gram-negative bacteria. The engagement of TLR4 with LPS leads to the activation of TLRs initiates TLR signaling cascade, leading to the activation of NF-κB (57), which in turn induces the transcription of proinflammatory cytokines, chemokines, and upregulation of cell surface molecules. The critical role of the canonical pathway of NF-κB in EAE was shown when this pathway of NF-κB signaling was targeted in astrocytes (24, 58). Our studies show that TGF-β signaling in the brain is activated by these innate immune triggers and may have a critical role in setting the conditions for local immune disease.

Our study investigates an important but often neglected aspect of autoimmune encephalomyelitis by exploring the conditions that facilitate the accumulation of autoimmune T cells in the CNS. We propose that TGF-β1 has a key role in creating a permissive environment several days before the onset of clinical disease in EAE by activating TGF-β signaling first in CNS cells and then in infiltrating T cells. The influence of TGF-β signaling in glial cells of the nervous system on the immune system is striking here. Inhibition of TGF-β signaling counteracts these events and therefore may have therapeutic benefits in the early phases of autoimmune inflammation in the CNS.

**Methods**

**Mice.** The following transgenic mouse lines were used: C57BL/6 (The Jackson Laboratory), SBE-luc (line T9-7BA on the C57BL/6 genetic background, “responder” mice screened by detectable luciferase activity (31, 32), GFAP–pTGF-β1 (line T64B on the C57BL/6 genetic background, contain a constitutively active mutant of porcine TGF-β1 under control of a GFAP promoter) (36), SBE-lucRT (line T182-103 express a fusion protein containing luciferase, BFP, and thymidine kinase (33) under the control of SBE promoter), and GFAP-luc (26). SBE-lucRT and GFAP-luc mice, originally generated on the FVB/N genetic background, were crossed with C57BL/6J-Tyr transgenic mice, and F1 offspring were used for experiments. Mice were between 8 and 12 weeks of age when experiments were initiated. All animal care and use was in accordance with institutional guidelines and approved by the VA Palo Alto Committee on Animal Research.

**EAE induction, assessment, and IN-1130 treatment.** MOG35–55 peptide (MEV-GWYRSPFSRVHLYRNGK) was synthesized by the Stanford Protein and Nucleic Acid Biotechnology Facility and purified by high-performance liquid chromatography to greater than 95% purity. Mice were immunized...
Splenocytes were isolated and cultured in vitro with IL-6, IL-12, IL-17, and IL-23. Cytokine levels were determined using specific ELISA kits for the corresponding cytokines according to the manufacturers’ protocols (eBioscience and BD Biosciences).

Quantitative real-time PCR. Total RNA was isolated from spinal cords using Absolutely RNA Miniprep Kit (Stratagene) and converted to cDNA using SuperScript II RNase H- Reverse Transcriptase (Invitrogen) for first-strand cDNA synthesis. The cDNA product was used for real-time quantitative PCR using a high-speed thermal cycler (LightCycler3; Roche Diagnostics) and detection of product by SYBR green I (QIAGEN). PCR primers were as follows: for detection of TGF-β1, forward 5′-CTTGGAAATCAACGGG-3′, reverse 5′-CAGAAGTTGGCATGTT-3′; for detection of IL-6, forward 5′-ACAACCCGCGCTTCCCTAC-3′, reverse 5′-TCCAGATTCTCCAGAGA-ACA-3′; and for detection of IL-23, forward 5′-AATGGGCGCGTATCCTAC-3′, reverse 5′-GGAGTGTGGAAGTGGCT-3′ (Operon Biotechnologies Inc.). Melting curves confirmed that only 1 product was amplified. Specific cDNA was quantified with a standard curve based on known amounts of amplified β-actin fragment and expressed as fold increase over baseline levels obtained from nonimmunized control mice.

Isolation of T cells from CNS tissue. Brains and spinal cords were obtained from mice with EAE after PBS perfusion and were ground through a cell strainer and digested with 300 U/ml clostridial collagenase (type V) to obtain a single-cell suspension. CNS-derived cells were washed and resuspended in 30% Percoll, underlayered with 70% Percoll, and centrifuged at 500 g for 20 minutes. CNS mononuclear cells were collected from the 30%/70% interface.

Flow cytometric analysis. Cells obtained from spinal cords or spleens were washed and incubated with anti-CD16/32 to occupy Fc receptors and prevent nonspecific binding. All cells were first stained with anti-CD4-FITC. They were then fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences) and incubated with PE-conjugated antibodies against IL-4, IFN-γ, Foxp3, and IL-17 (BD Biosciences) for intracellular staining. To determine the expression of RFP, cells isolated from SBE-lucRT mice were stained with anti-RFP-FITC (Abcam Inc.) and PE-conjugated antibodies. Stained cells were analyzed with a BD FACScan flow cytometer, and data were analyzed with FlowJo 8.5.2 (Tree Star Inc.).

Statistics. Data were expressed as mean ± SEM. Statistical analyses were performed with Prism 4.03 software (GraphPad Software). Means between 2 groups were compared with 2-tailed, unpaired Student’s t test; comparisons of means from multiple groups with 1 control were analyzed with 1-way ANOVA and Dunnett’s or Tukey’s post hoc test. Comparing distributions of FACS data was performed with FlowJo Population Comparison Algorithm. A value T(X) was obtained and was used to estimate the probability that a test population was different from a control population. When T(X) = 0, 2 histograms are indistinguishable (P = 0.5); T(X) > 4 indicates that 2 distributions are different with P < 0.01 (99% confidence).

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