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Neuron-mediated generation of regulatory T cells from encephalitogenic T cells suppresses EAE

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Neurons have been neglected as cells with a major immune-regulatory function because they do not express major histocompatibility complex class II. Our data show that neurons are highly immune regulatory, having a crucial role in governing T-cell response and central nervous system (CNS) inflammation. Neurons induce the proliferation of activated $CD4^+$ T cells through B7-CD28 and transforming growth factor (TGF)-β1–TGF-β receptor signaling pathways, resulting in amplification of T-cell receptor signaling through phosphorylated ZAP-70, interleukin (IL)-2 and IL-9. The interaction between neurons and T cells results in the conversion of encephalitogenic T cells to $CD25^+$TGF-$\beta1^+$CTLA-$4^+$$FoxP3^+$ T regulatory (T_{reg}) cells that suppress encephalitogenic T cells and inhibit experimental autoimmune encephalomyelitis. Suppression is dependent on cytotoxic T lymphocyte antigen (CTLA)-4 but not TGF-β1. Autocrine action of TGF-β1, however, is important for the proliferative arrest of T_{reg} cells. Blocking the B7 and TGF-β pathways prevents the CNS-specific generation of T_{reg} cells. These findings show that generation of neuron-dependent T_{reg} cells in the CNS is instrumental in regulating CNS inflammation.

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The role of neurons in the regulation of CNS inflammation has been addressed in only a few earlier studies—mainly through their interaction with residual antigen-presenting cells (APCs), namely glial cells, in the CNS[1,2]. Collectively, these reports contribute to the notion that neurons might indirectly function as T-cell regulators through control of CNS-specific APCs. Even less is known about direct communication between T cells and neurons, but cells from the immune system have been implicated in protecting neurons from degeneration[3–5]. These studies suggested the existence of an extensive cross-talk between the immune system and the CNS.

Neurons can induce T-cell apoptosis[6], and T cells have been shown to bind to neurons through leukocyte function-associated antigen 1 (LFA-1; on T cells) and intercellular adhesion molecule 5 (ICAM-5; on neurons)[7,8]. Further supporting an immune-regulatory function of neurons, we reported that neurons produce the anti-inflammatory cytokine TGF-β1, which is associated with resistance to experimental autoimmune encephalomyelitis (EAE)[9–12]. In addition, recovery from EAE in mice was correlated with upregulation of B7.1 and TGF-β1 in neurons—both of which are vital to the regulation of the immune response[12]. Together, these data suggest a role for neurons in the regulation of CNS inflammation.

TGF-β1 is a crucial cytokine in the regulation of T cell–mediated immune responses and in the induction of immune tolerance[13,14]. When TGF-β1 signaling was abolished in T cells[15,16], mice developed unchecked T-cell proliferation as well as inflammatory and autoimmune-like diseases. The suppressive role of TGF-β has also been established in EAE, in which *in vivo* blocking of TGF-β at early stages was reported to accelerate and aggravate the disease[17]. These reports show the importance of TGF-β–dependent signaling in T-cell activation and tolerance *in vivo*. The role of the B7 family (B7.1 and B7.2) as costimulatory molecules is well documented and is important in the activation and termination of the T-cell response through binding to CD28 or CTLA-4, respectively, thus inducing a secondary signal after engagement of the T-cell receptor (TCR)[18].

Here, we investigated how neurons interact with T cells and examined the potential role of the neuronal B7 interaction with ligands on T cells in the absence of major histocompatibility complex (MHC) class II–TCR signaling. Further, we investigated the role of neuronal TGF-β1 in the outcome of the T-cell response. To study the interaction of neurons and encephalitogenic T cells, we used an *in vitro* coculturing system and extended the findings *in vivo* using active and adoptive transfer EAE. Our data show that neurons have a crucial role in the regulation of the T-cell response.

RESULTS

T cells induce upregulation of TGF-β1 and B7 in neurons

We first investigated the circumstances under which neurons upregulate TGF-β1 and B7 and asked whether these molecules influence T-cell regulation in the CNS. Cultured neurons expressed *Tgfb1* (which encodes TGF-β1) and *Cd80* (which encodes B7.1) mRNA and corresponding proteins (**Fig. 1a–d**). Neuronal production of TGF-β1 was visualized by confocal microscopy *in vitro* and by immunohistochemistry *in vivo* in the CNS of mice with EAE at day 7 after immunization (**Fig. 1e,f**). In addition, neurons in culture secreted a substantial amount of TGF-β1 into the culture medium. When neurons were cocultured with T cells, however, TGF-β1 levels in the culture medium dropped, suggesting that neuronal TGF-β1 is

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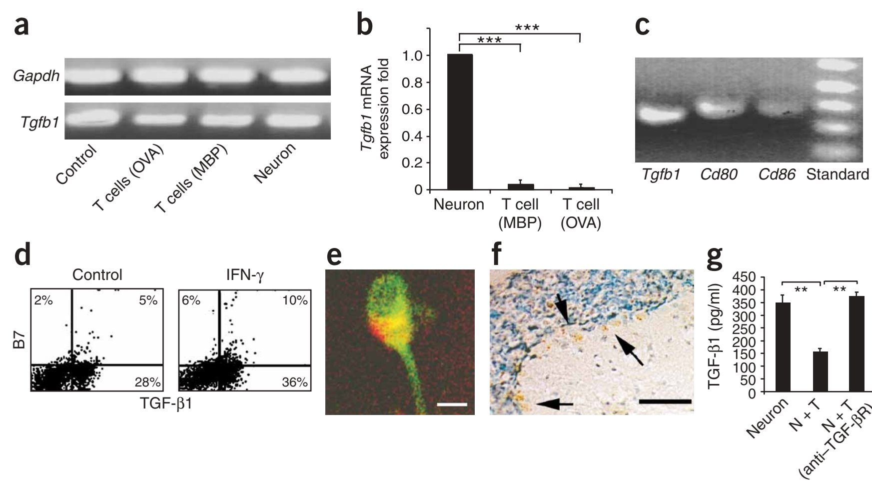

Figure 1 Neurons produce TGF-β1 and express B7 molecules. (**a**,**b**) Quantitative expression of *Tgfb1* mRNA in nonstimulated neurons, in comparison to MBP- and OVA-specific T-cell lines (after 72 h of stimulation) using real-time PCR. Fibroblasts served as a positive control and *Gapdh* as a loading control. (**c**) Neuronal expression of *Cd80*, *Cd86* and *Tgfb1* mRNA, as determined by real-time PCR. (**d**) FACS analysis of B7 (B7.1 and B7.2) and TGF-β1 expression on neurons before and after stimulation with recombinant IFN-γ. Numbers in quadrants refer to percentages of gated cell populations. (**e**) Confocal image showing intracytoplasmic staining of TGF-β1 (red) and neurofilament-200 (green) in a 3-d-old neuronal culture. Scale bar, 10 μm. (**f**) Immunohistochemistry staining of TGF-β1 in a brain section of a mouse with EAE. Arrows indicate parenchymal neurons in the cerebellum expressing intracellular TGF-β1 (brown color). Scale bar, 100 μm. (**g**) The amount of TGF-β1 secreted in culture supernatants by neurons and neurons cocultured with an activated MBP T-cell line (ratio, 1:1) analyzed using ELISA. Assay was performed with or without blocking antibody to TGF-βR (anti–TGF-βR) on T cells before coculture. N, neurons; T, T cells. Data are mean ± s.d., $n = 3$, $^{**}P < 0.01$, $^{***}P < 0.001$.

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taken up by T cells. Blocking the TGF-β receptor (TGF-βR) on T cells before coculture restored TGF-β1 levels in the culture medium (**Fig. 1g**).

Neuron–T cell interaction resulted in upregulation of *Cd80*, *Cd86*, *Icam1*, *Tgfb1* and *Tgfbr2* mRNA (encoding B7.1, B7.2, ICAM-1, TGF-β1 and TGF-βR, respectively) in neurons (**Fig. 2a**,**b**). In addition, the percentage of neurons expressing B7.1, B7.2, TGF-β1, TGF-βR and ICAM-1 was also significantly increased upon coculture with T cells (**Fig. 2c**). Blocking production of interferon (IFN)-γ and tumor necrosis factor (TNF)-α in T cells before coculture inhibited upregulation of these molecules on neurons (**Fig. 2c**). When recombinant IFN-γ and TNF-α were added to the neurons in culture, neuronal expression of B7.1, B7.2, TGF-β1, TGF-βR and ICAM-1 was induced (**Fig. 2d**). Together, these data suggest that production of IFN-γ and TNF-α by encephalitogenic T cells triggers induction of these molecules on neurons. Furthermore, T-cell interaction with neurons results in significantly higher neuron survival, suggesting that this interaction is protective for neurons (**Fig. 2e**).

Neurons increase T-cell proliferation and TCR signaling

To investigate how the neuron–T cell interaction affects the T-cell response, we used syngeneic-activated encephalitogenic T-cell lines in cocultures with neurons, as only activated T cells cross the blood-brain barrier[3,19,20]. T-cell proliferation was significantly induced by neurons and is dependent on cell-to-cell contact (**Fig. 2f**,**g**). This finding contrasts with the classical pathway of antigen-induced proliferation by professional APCs (**Fig. 2f**). Neuron-induced proliferation of activated T cells was associated with significant augmentation of TCR signaling, as determined by increases in phosphorylated ZAP-70, IL-2 and IL-9 (**Fig. 2h**). TCR signaling was independent of MHC class II, as neurons did not express MHC II and proliferation was independent of antigen (data not shown).

Neuronal TGF-β1 and B7, crucial for T-cell proliferation

The fact that expression of B7 and TGF-β1 in neurons is induced by T cells suggested that these molecules might be important for the outcome of the T-cell response. Blocking these signaling pathways showed that the TGF-β1–TGF-βR pathway has a crucial role in the T-cell proliferative response, as proliferation was prevented using an antibody to TGF-β1 (**Fig. 3a**). T-cell proliferation was only prevented by blocking TGF-βR on T cells, but not on neurons (**Fig. 3a**), suggesting that neuronal TGF-β1 signals through TGF-βR on T cells. As blocking B7 molecules also abolished the proliferative response (**Fig. 3b**), B7-CD28 signaling was also crucial in the neuronal–T cell interaction.

Neuronal generation of $CD4^+CD25^+$TGF-$\beta1^+$CTLA-4^+ T cells

To further investigate the importance of the TGF-β1–TGF-βR signaling pathway in T cells, we determined levels of *Tgfb1*, *Tgfbr2* and *Smad3* (encoding Smad3, an intracellular signaling protein involved in this pathway) mRNA using real-time PCR. Upon interaction with neurons, mRNA expression levels for all these proteins were significantly elevated in encephalitogenic T-cell lines (**Fig. 3c** and **Supplementary Fig. 1** online). For T cells to acquire membrane-bound TGF-β1, cell-to-cell contact with neurons was required (**Fig. 3d**). The number of TGF-$\beta1^+$ T cells was increased 12 h after coculture with neurons, followed by upregulation of CTLA-4 after 24 h (**Fig. 3e**); this population of cells (TGF-$\beta1^+$ CTLA-4^+ T cells) increased after 96 h (data not shown). We found that, after interaction with neurons, encephalitogenic T cells upregulate FoxP3, a transcription factor known to be important in regulatory function of naturally occurring thymus-derived regulatory $CD25^+CD4^+$ T_{reg} cells (**Fig. 3c**,**f**). These T cells also significantly upregulated other activation markers (CD25, CD69, CD28 and IL-2), and $CD25^+$ T cells were enriched among the TGF-$\beta1^+$ population ($CD25^+$TGF-$\beta1^+$ T cells; **Fig. 3f**). To investigate whether expression of TGF-β1 expression on T-cell membranes was associated with their proliferative response, we carried out Ki-67 staining; the proliferative population was largely TGF-$\beta1^-$ (Ki-67^+TGF-$\beta1^-$; **Fig. 3g**).

We then asked whether neurons could induce proliferation of naive T cells, as, theoretically, these cells could interact with neurons as a result of a physical insult to the CNS. We found that neurons did not induce proliferation of naive T cells nor did they induce upregulation of CD69, but they did induce $CD25^+$TGF-$\beta1^+$CTLA-4^+ phenotypes (**Fig. 4a**,**b**). Depletion of $CD4^+CD25^+$ T_{reg} cells from purified $CD4^+$ T cells resulted in similar findings (data not shown),

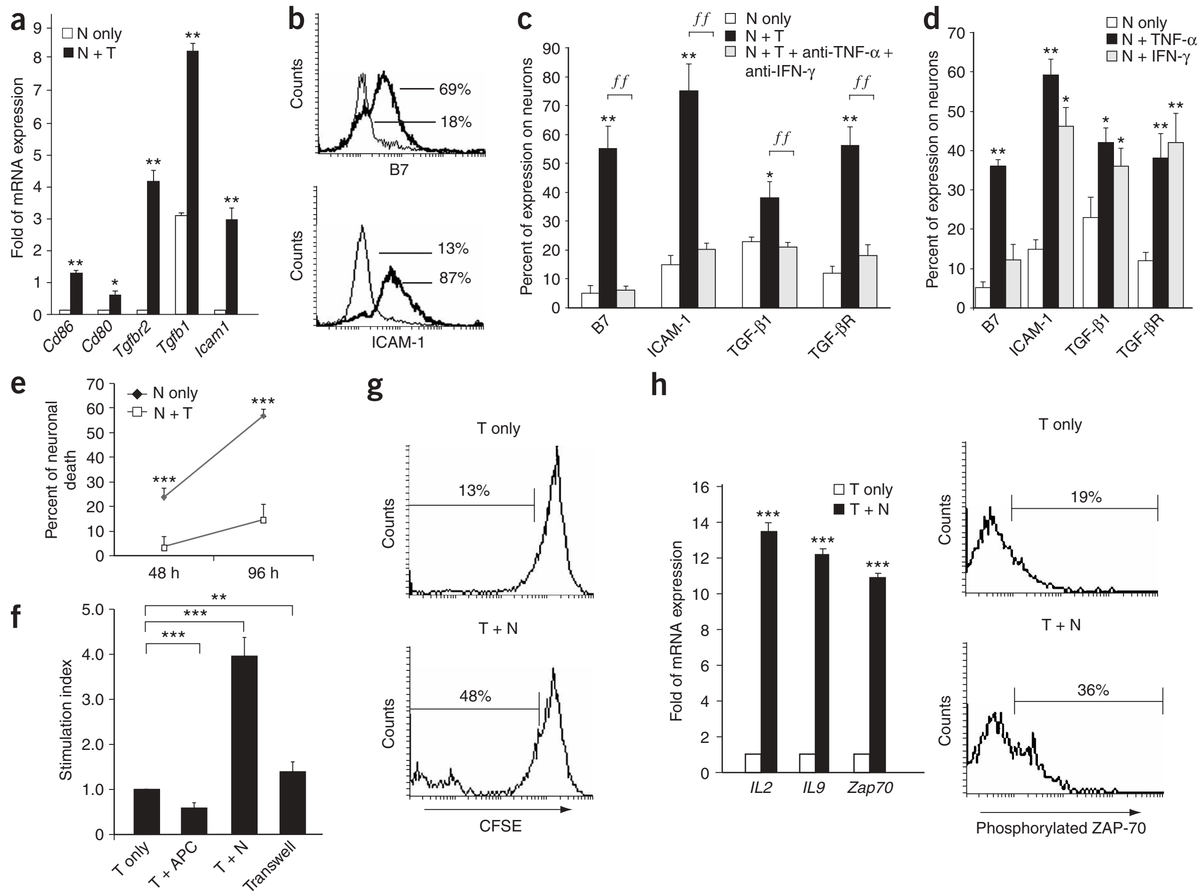

Figure 2 T cells upregulate B7.1, B7.2, TGF-β1, TGF-βR and ICAM-1 on neurons, and neurons induce activation of effector T cells. Neuronal expression of B7.1, B7.2, ICAM-I, TGF-β1 and TGF-βR, detected by: (**a**) real-time PCR before and after coculture with T cells, (**b**) FACS before (light curve) and after coculture with T cells (dark solid curve), (**c**) with or without T-cell coculture, after blocking T-cell cytokines using IFN-γ–specific antibody (20 μg/ml) and TNF-α–specific antibody (20 μg/ml) before coculture and (**d**) after stimulation with recombinant IFN-γ and TNF-α. (**e**) Trypan blue staining was used to detect the ratio of dead neurons before and after coculture with T cells. (**f**) A T-cell line was activated by MPB_{89-101} with irradiated syngeneic APCs for 48 h. T cells and neurons were cocultured (ratio, 1:1) for another 24 h. Direct cell-to-cell contact was prevented using a transwell system. Activated T-cell lines, alone or cocultured with antigen-irradiated syngeneic APCs, served as controls. Proliferative responses were determined by a [^{3}H]-thymidine incorporation assay. (**g**) CFSE labeling of activated T cells analyzed 72 h after coculturing with neurons. (**h**) Left, expression of *IL2*, *IL9* and *Zap70* mRNA (encoding IL-2, IL-9 and ZAP-70, respectively) was determined using real-time PCR in encephalitogenic T cells before and after coculture with neurons. Right, expression of phosphorylated ZAP-70 on activated T cells and T cells after coculture with neurons was detected by FACS. N, neurons; T, T cells. Data are mean ± s.d., $n = 3$, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{ff}P < 0.01$.

arguing against the possibility of enrichment of existing $CD4^+CD25^+$ T_{reg} cells.

CTLA-4 mediates T-cell regulatory function

To address the function of $CD25^+$TGF-$β1^+$CTLA-4^+ T cells, activated T cells were used as responder cells, whereas neuron-induced $CD25^+$TGF-$β1^+$CTLA-4^+ T cells served as suppressor cells. Responder cells proliferated well. Suppressor cells did not proliferate, and they suppressed responder-cell proliferation (**Fig. 4c**). We investigated whether the suppressive function is dependent on TGF-β1 and/or CTLA-4, as both these signaling pathways have been suggested to be important for function of T_{reg} cells. Blocking CTLA-4 rescued responder T-cell proliferation, showing that the suppressive function of these cells depends on CTLA-4. Lack of proliferative response in suppressor cells, however, did not depend on CTLA-4. In contrast, blocking TGF-β1 reversed proliferative arrest of these suppressor cells but did not alter their suppressive function (**Fig. 4d**). Hence, TGF-β1 exerts an autocrine effect, inhibiting proliferation of neuron-induced $CD25^+$TGF-$β1^+$ CTLA-4^+ T_{reg} cells.

$CD25^+$TGF-$β1^+$CTLA-4^+ T_{reg} cell enrichment in CNS of EAE mice

To determine whether $CD25^+$TGF-$β1^+$CTLA-4^+ T_{reg} cells are also generated by neuronal induction *in vivo*, we isolated CNS-infiltrating cells from mice with active EAE. $CD4^+$TGF-$β1^+$ T cells are highly enriched in the CNS, compared to lymphoid organs (**Fig. 5a**). We stained isolated CNS-infiltrating cells for membrane-bound TGF-β1, and compared membrane-bound TGF-β1 to intracellular production of TGF-β1 at different time points of EAE. Membrane-bound TGF-$β1^+$ $CD4^+$ T cells were present in the CNS at day 7 after immunization, increased at day 18 after immunization and subsequently declined. The number of T cells producing intracellular TGF-β1 was initially low, but increased substantially at day 25 after immunization, probably after uptake of TGF-β1 produced by neurons in the CNS (**Fig. 5b**). Upon interaction with neurons, T cells acquired the $CD25^+$TGF-$β1^+$CTLA-4^+ phenotype (**Fig. 5c**).

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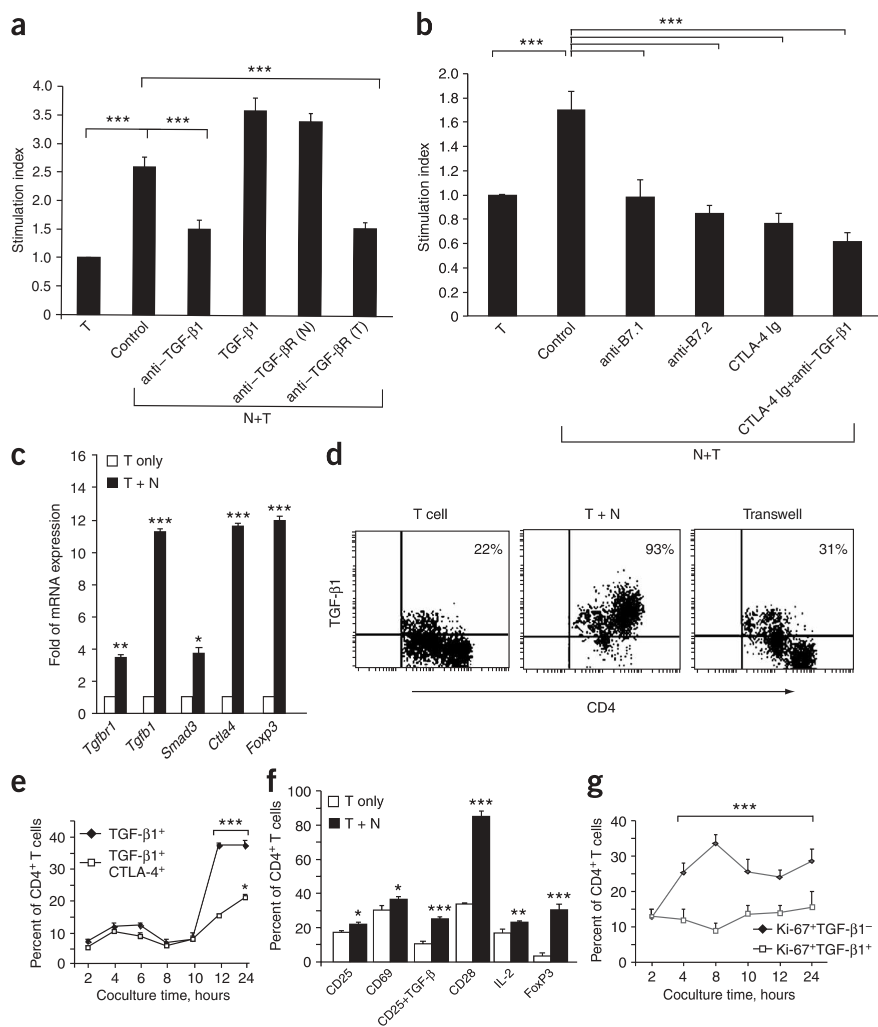

Figure 3 TGF-β1–TGF-βR and B7-CD28 signaling are essential for neuron-induced CD4+ T-cell proliferation and conversion to CD25+TGF-β1+ CTLA-4+ T cells. (**a**) Regulation of T-cell proliferation by modulating TGF-β signal. TGF-β1–specific (20 μg/ml), TGF-βR–specific (20 μg/ml) antibodies and recombinant TGF-β1 (0.05 μg/ml) were added to neuronal culture and TGF-βR–specific antibody to T cells before coculturing. (**b**) Blocking B7 signal inhibits T cell proliferation. B7.1-specific Fab antibody (10 μg/ml), B7.2-specific Fab antibody (10 μg/ml), CTLA-4–specific Ig (10 μg/ml), combined CTLA-4–specific Ig with TGF-β–specific antibody or isotype-match Ig were added to neuronal culture before coculturing. (**c**) Expression of *Tgfbr1*, *Tgfb1*, *Smad3*, *Ctla4* and *Foxp3* mRNA (encoding TGF-βR, TGF-β1, Smad3, CTLA-4 and FoxP3, respectively) was determined by real-time PCR in activated T cells versus T cells after coculture with neurons. (**d**) Expression of membrane-bound TGF-β1 of T cells (left), T cells cocultured with neurons (middle) or T cells in a transwell plate with neurons (right) was determined by FACS. Numbers in quadrants refer to percentages of gated CD4+ T-cell populations. (**e**) Percentage of TGF-β1+ and TGF-β1+CTLA-4+ of total CD4+ T cells was determined at different time points during culture of T cells with neurons. (**f**) Percentage of CD25+, CD69+, CD25+TGF-β1+, CD28+, IL-2+ and FoxP3+ of total CD4+ T cells before and after coculture with neurons. (**g**) Percentage of Ki-67+TGF-β1+ and Ki-67+TGF-β1− cells of total CD4+ T cells at different time points during coculture with neurons. N, neurons; T, T cells. Data are mean ± s.d., $n = 3$, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

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We transferred *in vitro*–enriched CD4+GFP-MBP$_{89-101}$-reactive T cells to irradiated mice with or without splenectomy to track their fate *in vivo*. We aimed to ascertain whether homing of encephalitogenic GFP+ T cells to the spleen is required[21] for the phenotypic changes, and/or whether their conversion to the T_{reg} phenotype takes place in the CNS. Three days after transfer, we isolated CNS-infiltrating T cells and analyzed them for the T_{reg} phenotype. The number of GFP+ T cells was markedly higher in the CNS as compared to the spleen (42.0 ± 3% versus 14.5 ± 1.5%, respectively). No differences were observed between mice with or without splenectomy with regard to CD25+TGF-β1+CTLA-4+ T-cell homing to the CNS. We confirmed that encephalitogenic GFP+ T cells infiltrate the CNS without prior homing to the spleen (**Fig. 5d**). In the CNS, GFP+ T cells acquired the T_{reg} phenotype, that is, they expressed membrane-bound TGF-β1 (data not shown), and a large number of these cells were also FoxP3+ (**Fig. 5d**).

We then investigated the *in vivo* role of the molecules involved in cross-talk between T cells and neurons *in vitro*, and examined the generation of T_{reg} cells *in vivo*. We studied the capacity of GFP+ T cells to infiltrate the CNS and their phenotypic changes before and after blocking signaling molecules. Combined IFN-γ–specific and TNF-α–specific antibody treatment, at the time of T-cell transfer, severely reduced infiltration of GFP+ T cells to the CNS compared with control (20×10^4 versus 5×10^4, respectively), indicating that these cytokines have an important role in the homing of autoreactive T cells to the CNS. To address CNS-specific effects, we blocked TGF-β1 and B7 signaling pathways 1 d after T-cell transfer. At 3 d after transfer, the conversion of encephalitogenic T cells to CD4+TGF-β1+ and CD4+FoxP3+ T_{reg} phenotypes was inhibited in the group receiving combined TGF-β1–specific antibody and CTLA-4–specific immunoglobulin (**Fig. 5e**). Blocking these signaling pathways did not affect the homing of encephalitogenic GFP+ T cells to the CNS, as the CNS of mice that received the blocking agents contained T-cell numbers similar to those of the control mice (2.3×10^5 versus 2.1×10^5, respectively). These data show the crucial role of IFN-γ and TNF-α (produced by T cells) and TGF-β and B7 (produced by neurons) for their cross-talk, resulting in the generation of T_{reg} cells *in vivo* in the CNS (**Supplementary Figs. 1** and **2** online).

Neuron-induced and CNS-derived T_{reg} cells suppress EAE

To investigate the functional properties of T_{reg} cells *in vivo*, we generated T_{reg} cells *in vitro* by coculturing encephalitogenic T cells with neurons or by isolating CNS-derived T_{reg} cells from CNS-infiltrating cells. We then purified CD4+TGF-β1+ and CD4+TGF-β1− T cells and transferred them along with encephalitogenic T-cell lines into mice to induce EAE through adoptive transfer. Both *in vitro*– and *in vivo*–derived CD4+TGF-β1+ T_{reg} cells prevented induction of EAE (**Fig. 6** and **Supplementary Table 1** online). The

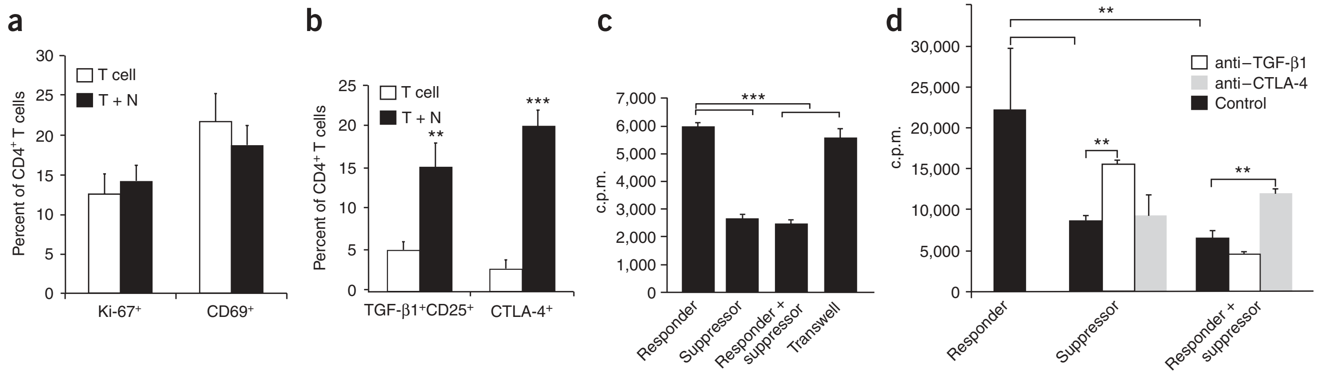

Figure 4 Neuron-generated CD25+TGF-β1+CTLA-4+ T cells exert regulatory function through CTLA-4. (**a**) FACS analysis of percentage of Ki-67+ and CD69+ of naive CD4+ T cells before and after coculture with neurons. (**b**) FACS analysis of percentage of TGF-β1+CD25+ and CTLA-4+CD4+ T cells before and after coculture with neurons. Suppressive function was measured using [^{3}H]-thymidine incorporation in coculture assay. Responder T cells refer to activated T-cell lines. Suppressor T cells refer to activated T cells after coculture with neurons for 24 h to allow generation of CD25+TGF-β1+CTLA-4+ T cells. (**c**) Responder and suppressor T cells were cultured separately and also cocultured in a 1:1 ratio. Cells were activated identically with antigen and irradiated APCs; transwells were used for preventing cell-to-cell contact (**d**) TGF-β1–specific antibody (20 μg/ml) or CTLA-4–specific antibody (20 μg/ml) were added to suppressor T cells for 30 min before coculture. T cells were activated by plate-bound CD3-specific antibody in a 1:1 ratio. N, neurons; T, T cells. Data are mean value (counts per minute (c.p.m.) ± s.d.), $n = 3$, $^{**}P < 0.01$, $^{***}P < 0.001$.

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suppressive function of neuron-induced T_{reg} cells was antigen independent (**Fig. 6b**). These data suggest that, upon entering the CNS, encephalitogenic T cells convert to CD4+TGF-β1+ T_{reg} cells, which have a suppressive effect on CNS inflammation.

DISCUSSION

Our study addressed the direct immune-regulatory role of neurons on T cells. Although it has been established that there is cross-talk between cells of the immune system and the CNS[22–27], information on the direct neuron–T cell interaction is limited. Previously, we reported that upregulation of TGF-β1 and B7 by neurons is associated with recovery and resistance to EAE[9–12]. Collectively, these data indicated that neuron–T cell communication could be bidirectional, suggesting that neurons might directly affect the immune system, including T cells. Hence, the hypothesis that potential neuronal B7 ligation with its receptors on T cells, in the absence of MHC class II signaling, could lead to differential regulation of T-cell response served as the basis for the current study.

Here, we present data showing that neurons have a crucial role in the regulation of T-cell response and CNS inflammation. We report that neurons induce proliferation of encephalitogenic CD4+ T cells through the B7-CD28 and TGF-β1–TGF-βR signaling pathways, independent of MHC class II, as T-cell proliferation was not antigen specific. The resulting T-cell proliferation is associated with amplification of TCR signaling through phosphorylated ZAP-70, a tyrosine kinase crucial for efficient stimulation of T cells through TCR-CD3 signaling[28], as well as upregulation of IL-2 and IL-2 receptor α chain (CD25). Further, supporting the involvement of the TGF-β1–TGF-βR pathway, we showed that, upon interaction with neurons, T cells upregulate Smad3, an intracellular protein important for successful signaling through TGF-β1–TGF-βR[29]. Moreover, this leads to upregulation of IL-9, consistent with a report that production of IL-9 is triggered by TGF-β1 (ref. 30).

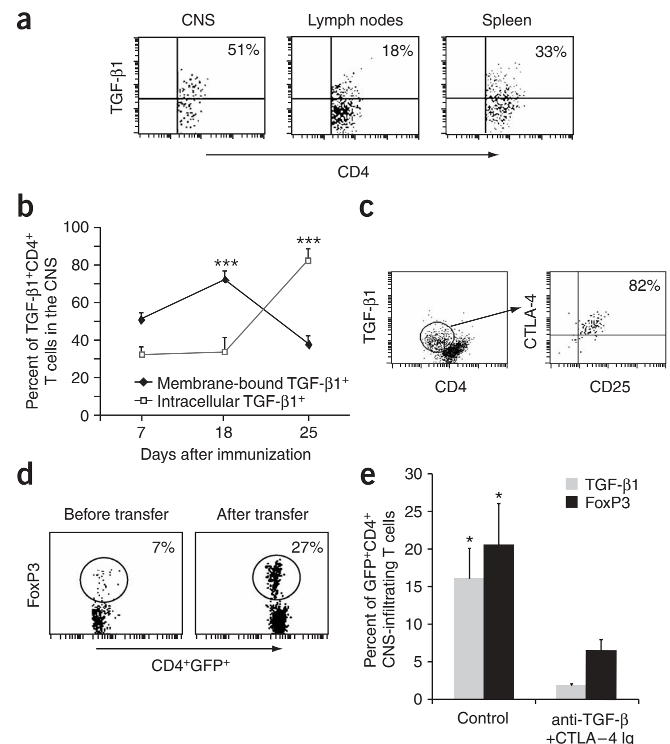

Figure 5 *In vivo* enrichment of CD4+TGF-β1+FoxP3+ T cells in the CNS of mice with EAE requires TGF-β and B7 signaling. (**a**) Membrane-bound TGF-β1+ T cells (gated with CD3+CD4+) isolated from CNS or lymphoid organs 7 d after induction of active EAE in C57BL/6 mice. Numbers in quadrants refer to percentages of gated cell populations. (**b**) Percentage of membrane-bound versus intracellular TGF-β1+ T cells in CNS-infiltrating cells of active EAE at 7, 18 and 25 d after immunization. (**c**) Right, percentage of CD25+CTLA-4+ among gated CD4+TGF-β1+ T cells (left) isolated from CNS-infiltrating T cells. (**d**) MBP_{89-101}-reactive CD4+GFP+ T cells were transferred to irradiated and splenectomized mice. Percentage of FoxP3+CD4+GFP+ T cells in isolated infiltrating cells from the CNS of mice 3 d after *in vivo* adoptive transfer. (**e**) Comparison between mice receiving TGF-β1–specific antibody (50 μg/mouse) and CTLA-4–specific immunoglobulin (100 μg/mouse) with control group the day after MBP_{89-101}-reactive CD4+GFP+ T-cell transfer. Mean value ± s.d., $n = 3$, $^{*}P < 0.05$, $^{***}P < 0.001$.

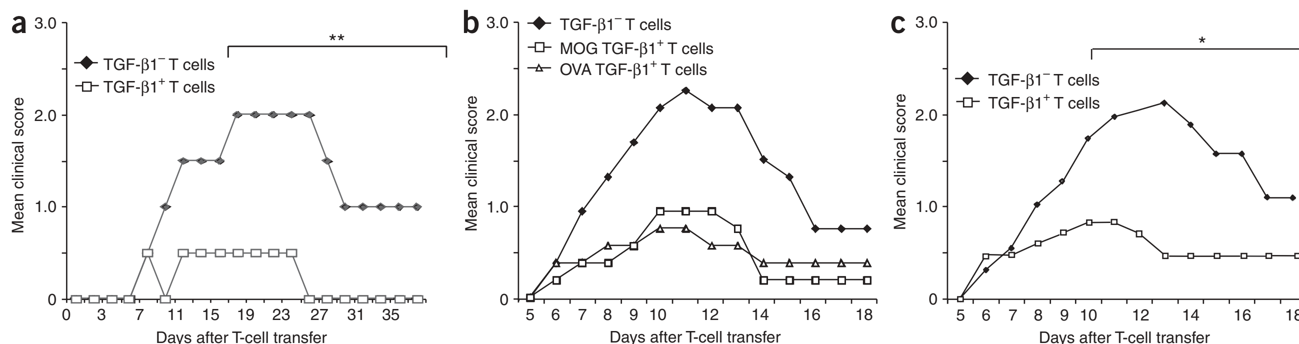

Figure 6 Neuron-induced and CNS-derived CD4$^+$TGF-β1$^+$ T cells suppress EAE. C57BL/6 mice were irradiated and adoptive transfer EAE was induced with intravenous transfer of MOG$_{35-55}$-reactive T cells. (**a**) CD4$^+$TGF-β1$^+$ and CD4$^+$TGF-β1$^-$ T cells were purified from encephalitogenic T cells cocultured with neurons *in vitro* and were cotransferred with a MOG$_{35-55}$-reactive T-cell line ($n = 6$). (**b**) MOG$_{35-55}$-reactive CD4$^+$TGF-β1$^+$ T cells and OVA-reactive CD4$^+$TGF-β1$^+$ T cells were purified from activated T cells cocultured with neurons *in vitro* and then they were cotransferred with a MOG$_{35-55}$-reactive T-cell line ($n = 5$). (**c**) B10.RIII mice were irradiated and adoptive transfer EAE was induced with intravenous transfer of MBP$_{89-101}$-reactive T-cell line. CD4$^+$TGF-β1$^+$ T cells were isolated from *in vivo* CNS-infiltrating T cells from actively induced EAE mice (18–20 d after immunization) and then cotransferred with MBP$_{89-101}$-reactive T-cell line intravenously to B10.RIII-naive mice the same day ($n = 6$). Graphs show mean clinical score ± s.d., $*P < 0.05$, $**P < 0.01$.

Notably, neuron–T cell interaction results in the generation of T cells with a stable CD25$^+$TGF-β1$^+$CTLA-4$^+$ phenotype from committed encephalitogenic CD4$^+$ T cells. Neuron-induced proliferation of T cells and conversion to the CD25$^+$TGF-β1$^+$CTLA-4$^+$ phenotype is dependent on cell-to-cell contact between neurons and T cells. This contact initially leads to triggering of TGF-β1 production by neurons. The TGF-β1 binds to surface TGF-βR on T cells and induces intracellular TGF-β1 and, consequently, CTLA-4 by T cells (**Supplementary Fig. 3** online). In line with our findings, the ability of TGF-β1– secreting dendritic cells to stimulate Tr1 cell proliferation has recently been reported[31]. We also showed that CD25$^+$TGF-β1$^+$CTLA-4$^+$ T cells have a regulatory function, both *in vitro* and *in vivo*, as they suppress proliferation of encephalitogenic CD4$^+$ T cells and inhibit progression of EAE. This finding is in agreement with a previous report that TGF-β1 can induce naive CD4$^+$CD25$^-$ T cells to express cell-surface TGF-β1 and become CD4$^+$CD25$^+$ regulatory cells[32]. In contrast to reported thymus-derived CD4$^+$CD25$^+$ T$_{reg}$ cells[33] and peripherally converted CD4$^+$CD25$^+$ T$_{reg}$ cells from naive CD4$^+$CD25$^-$ T cells, we show here that CD25$^+$TGF-β1$^+$CTLA-4$^+$ T$_{reg}$ cells are converted from already-committed and activated encephalitogenic T cells. We also provide evidence that this occurs *in vivo* during CNS inflammation.

Neuron-induced T$_{reg}$ cells upregulate the transcription factor FoxP3, which is considered to be a specific marker for regulatory activity and is essential for the development and function of thymus-derived CD4$^+$CD25$^+$ T$_{reg}$ cells[33]. The *in vitro* suppressive function of CD25$^+$TGF-β1$^+$CTLA-4$^+$ T$_{reg}$ cells is dependent on CTLA-4, whereas TGF-β1 is not a key player in this suppression, in line with previous reports on the role of TGF-β in T$_{reg}$ cell function[34]. We report, however, that TGF-β1 is crucial for proliferation arrest in T$_{reg}$ cells, and this autocrine effect may be the mechanism limiting the expansion of T$_{reg}$ cells in the CNS (**Supplementary Fig. 3**).

The importance of CTLA-4 in delivering a negative signal to T cells is well documented[35,36]. The role of CTLA-4 in the regulatory effect of T$_{reg}$ cells, however, is not entirely clear. It has been suggested that a T cell–T cell interaction, involving B7 and CTLA-4, results in a suppressed T-cell response[37,38]. Our findings support this view, as the suppressive effect of neuronally converted CD25$^+$TGF-β1$^+$CTLA-4$^+$ T$_{reg}$ cells is completely abolished by blocking CTLA-4. The suppressive function of CTLA-4 in EAE has been previously documented; blocking CTLA-4 prevented acquired tolerance to EAE[39] and caused resistant mice to develop EAE[40].

Notably, neuron–T cell interaction leads to increased neuronal survival. We could not find any support for the involvement of nerve growth factor (NGF), but the neuronal survival effect could be mediated by production of other neurotrophic factors, which have been reported to prevent neurodegeneration[25].

We have shown that our *in vitro* findings are of direct relevance to the regulation of CNS inflammation *in vivo*. CD25$^+$TGF-β1$^+$CTLA-4$^+$ FoxP3$^+$ T$_{reg}$ cells become enriched in the CNS during the development of EAE. By using GFP-labeled encephalitogenic T cells, we established that these cells home to the CNS, where they acquire the regulatory phenotype. This is mediated through production of IFN-γ and TNF-α by T cells as well as signaling through TGF-β and B7 on neurons. Upon purification and transfer, these CNS-derived CD4$^+$TGF-β1$^+$ T$_{reg}$ cells suppress EAE. Neuron-induced CD25$^+$TGF-β1$^+$CTLA-4$^+$ T$_{reg}$ cells are also capable of preventing progression of EAE *in vivo*.

Our findings contribute to the understanding of how the CNS and the immune system interact and, more importantly, how inflammation in the CNS is likely to be regulated. The outcome of CNS inflammation could be greatly dependent on how the CNS-specific cells in general, and neurons in particular, interact with immune cells and shape their function. It is conceivable that nonmyelinated nerve endings that enter immune organs could also influence the T-cell response. Our findings showing that generation of neuron-dependent T$_{reg}$ cells in the CNS is instrumental in regulating CNS inflammation enhance understanding of the nature of CNS inflammatory diseases and may open new avenues for exploring therapeutic strategies in neurodegenerative diseases such as multiple sclerosis, Alzheimer and Parkinson disease.

METHODS

Mice. DO11.10 transgenic BALB/c, C57BL/6, B10.RIII and GFP B10.RIII (originally in NFR background[41], backcrossed to B10.RIII for 15 generations) strains of mice were bred and kept at the conventional animal facility of Lund University. Experiments were performed in accordance with the ethics committee in Malmö-Lund, Sweden.

Neuronal cultures. We coated culture plates with a poly-D-lysine solution (70 μg/ml). A 7-d-old mouse was decapitated and the cerebellum was carefully

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dissected out. We prepared a single-cell suspension by adding trypsin to a final concentration of 200 μg/ml. We added DNAse and FCS to final concentrations of 0.12% and 0.5%, respectively, and incubated for 8 min. The solution was dissociated into single cells using a fire-constricted Pasteur pipette. We assessed the ratio of dead neurons after 10 d in culture alone, and after 48-h and 96-h coculture with T cells, by trypan blue staining.

Electrophysiology. The cultured neurons expressed functional neuronal ion channels and receptors and were electrophysiologically viable (**Supplementary Methods** online). Step depolarization to –30 mV from a holding potential of –70 mV elicited fast inward sodium currents (183 ± 88 pA, $n = 7$). A saturating concentration of the inhibitory transmitter γ-aminobutyric acid (GABA) or the excitatory transmitter glutamate elicited currents of 391 ± 165 pA ($n = 5$) and 327 ± 123 pA ($n = 5$), respectively. The functional responses of the sodium, GABA- or glutamate-activated ion channels were similar for neurons cultured alone or in cocultures with T cells (data not shown) and is in accordance with appearance of ion channels during neuronal maturation.

Establishment of antigen-specific T-cell lines. We generated T-cell lines as previously described[42,43]. All T-cell lines were encephalitogenic, as they are capable of inducing chronic EAE upon adoptive transfer to naive mice[42,43]. We generated the GFP^+ T-cell line using GFP^+ B10.RIII mice and the ovalbumin$_{323-339}$ T-cell line using DO11.10 transgenic cells. In our experiments, we used all of these T-cell lines with similar results.

CFSE labeling. We labeled T cells with carboxy-fluorescein diacetate, succinimidyl ester (CFSE, Molecular Probes) using a method previously described[44].

MACS for sorting $CD4^+$ T cells. We used biotin-conjugated antibodies to Mac-1 (M1/70), B220 (RA3-6B2), pan natural killer cells (DX5) and CD8 (53-6.7) for negative separation. We used MACS Streptoavidin microbeads in the next step. For some experiments, after purification of $CD4^+$ T cells, we used biotin-labeled TGF-β1–specific antibody for sorting $CD4^+$TGF-$\beta1^+$ T cells. Cells were processed in an autoMACS machine (Miltenyi).

Quantitative real-time RT-PCR. We isolated total RNA using a Qiagen kit (Qiagen). Total RNA was reverse transcribed into cDNA, then amplified and quantified by detection of SYBR Green. We calculated relative mRNA expression using *Gapdh* gene expression as an endogenous reference. Primers used in this study are listed in **Supplementary Methods**.

FACS staining and evaluation. We performed these techniques according to the standard procedure (**Supplementary Methods**).

***In vitro* suppressive assay.** We used a coculture system and measured proliferation using a [^{3}H]-thymidine incorporation assay. We cocultured responder cells (antigen-activated T-cell lines or plate-bound CD3-specific antibody–activated T cells) in a 1:1 ratio with suppressor cells (activated T cells after coculture with neurons for 24 h; we checked for appearance of the TGF-$\beta1^+$ T-cell population). Responder and suppressor T cells were cocultured for another 72 h.

Induction of EAE and clinical evaluation. We used C57BL/6 and B10R.III mice for induction of active EAE with MOG_{35-55} and MBP_{89-101} peptides, respectively[42,43]. We used Complete Freund Adjuvant and pertussis toxin as adjuvant. We carried out adoptive transfer using irradiated mice (500 rad) C57BL/6 and B10R.II mice and injecting each in the tail vein with a cell suspension containing 2×10^6 MOG_{35-55} or 5×10^6 MBP_{89-101} T cells (for some experiments, the GFP^+ MBP_{89-101} T-cell line was used). Each mouse received cotransfer of either 1×10^6 TGF-$\beta1^+$ or TGF-$\beta1^-$ T-cells in 300 μl PBS (day 0). We purified TGF-$\beta1^+$ T cells and TGF-$\beta1^-$ T cells from coculture of neurons and T-cell lines. We carried out clinical scoring as previously described[42,43].

***In vivo* transfer and tracing of GFP^+ T cells.** We carried out splenectomy on B10.RIII mice 5 d before GPF^+ T-cell transfer. We irradiated each mouse (500 rad) and then injected each in the tail vein with a cell suspension containing 1×10^6 MBP_{89-101}-GFP^+ T cells. On the day of transfer (day 0) and 2 d later, we injected each mouse intraperitoneally with 500 ng pertussis toxin. On day 3, we purified CNS-infiltrating cells and analyzed them by FACS.

Preparation of CNS-infiltrating cells. At different time points after active EAE induction, we rapidly removed the brain, separated the hemispheres and isolated CNS-infiltrating cells as previously described[45].

Immunohistochemistry and intracytoplasmic staining. We performed staining according to standard procedures described in **Supplementary Methods**.

Statistical evaluations. We performed statistical evaluation using StatView software. We used Mann-Whitney tests to analyze differences in clinical scores. We analyzed the incidence of EAE disease using the χ^2 test. We used the Student unpaired *t*-test for other analyses.

Note: Supplementary information is available on the Nature Medicine website.

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COMPETING INTERESTS STATEMENT
The authors declare that they have no competing financial interests.

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