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*J Immunol* published online 15 October 2014
http://www.jimmunol.org/content/early/2014/10/15/jimmunol.1302401

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Supplementary Material
http://www.jimmunol.org/content/suppl/2014/10/15/jimmunol.1302401.DCSupplemental

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Regulatory T Cells in Central Nervous System Injury: A Double-Edged Sword

James T. Walsh,*§ Jingjing Zheng,*§ Igor Smirnov,*§ Ulrike Lorenz,‖,# Kenneth Tung,‡** and Jonathan Kipnis,*§§

Previous research investigating the roles of T effector (Teff) and T regulatory (Treg) cells after injury to the CNS has yielded contradictory conclusions, with both protective and destructive functions being ascribed to each of these T cell subpopulations. In this work, we study this dichotomy by examining how regulation of the immune system affects the response to CNS trauma. We show that, in response to CNS injury, Teff and Treg subsets in the CNS-draining deep cervical lymph nodes are activated, and surgical resection of these lymph nodes results in impaired neuronal survival. Depletion of Treg, not surprisingly, induces a robust Teff response in the draining lymph nodes and is associated with impaired neuronal survival. Interestingly, however, injection of exogenous Treg cells, which limits the spontaneous beneficial immune response after CNS injury, also impairs neuronal survival. We found that no Treg accumulate at the site of CNS injury, and that changes in Treg numbers do not alter the amount of infiltration by other immune cells into the site of injury. The phenotype of macrophages at the site, however, is affected: both addition and removal of Treg negatively impact the numbers of macrophages with alternatively activated (tissue-building) phenotype. Our data demonstrate that neuronal survival after CNS injury is impaired when Treg cells are either removed or added. With this exacerbation of neurodegeneration seen with both addition and depletion of Treg, we recommend exercising extreme caution when considering the therapeutic targeting of Treg cells after CNS injury, and possibly in chronic neurodegenerative conditions. The Journal of Immunology, 2014, 193: 000–000.

A cute injury to the CNS evokes cellular and molecular responses that lead to secondary neurodegeneration, a process of sustained neuronal degeneration (1). Accompanying this period of secondary degeneration is a coordinated immune response to the trauma, including chemotaxis of microglia to ATP released from the damaged cells (2) and directed migration of both the innate and adaptive immune cells to the injury site due to chemokine signals (3). The dogma that this infiltration of immune cells into the injury site was a detrimental response has been challenged by the finding that neuronal survival could be improved by boosting T cell activity rather than by its suppression (4–6), although the phenotype of protective T cells after CNS injury, and particularly the role of regulatory T (Treg) cells in this process, is still a matter of debate (7–10).

Naturally occurring Treg cells, which express the transcription factor Foxp3 (11–13), have been intensively studied for their ability to suppress adaptive immune responses (14–17). This subset of T cells, which develops with high avidity to self-Ags, is especially important in controlling autoimmunity (18). Therefore, it has been proposed that Treg cells mediate their actions by attenuating both protective and inflammatory postinjury immune responses, and thus either exacerbating (19) or ameliorating (20) neuronal degeneration. Despite these studies, the exact mechanism of their action in the injured CNS remains unclear.

Recently, the heterogeneity of macrophages has come to light, with two general classes being described as classically or alternatively activated (21). Although classically activated macrophages express high levels of proinflammatory cytokines such as TNF and IL-1b, and exhibit a robust respiratory burst (22), alternatively activated (tissue-building) macrophages express high levels of arginase-1 and several factors that play a role in promoting tissue homeostasis and recovery from insults (23). Several studies have shown the neuroprotective ability of alternatively activated macrophages in CNS injury (24–26), but what leads to, and sustains, this phenotype is unclear in the context of CNS trauma.

In this study, we show that the regulation of the T cell response to CNS injury is taking place in the draining deep cervical lymph nodes (dCLN) rather than at the site of injury. In line with this, surgical resection of the dCLN results in impaired neuronal survival. We show that removal of Treg cells that leads to exaggerated response of T effector (Teff) cells is associated with reduction in alternatively activated macrophages at the site of injury and leads to impaired neuronal survival. Exogenous supply of activated Treg cells, however, results in suppression of neuroprotective IL-4–producing T cells, and consequently also results in suppression of alternatively activated macrophages at the site of injury. Thus, both depletion and addition of Treg cells are detrimental for

Received for publication September 9, 2013. Accepted for publication September 20, 2014.

This work was supported by National Institute of Neurological Disorders and Stroke, National Institutes of Health Grant NS061973 (to J.K.).

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The online version of this article contains supplemental material.

Abbreviations used in this article: ATRA, all-trans retinoic acid; dCLN, deep cervical lymph node; DXs, diphtheria toxin; hCD2, human CD2; iTreg, Treg cells in vitro; RGC, retinal ganglion cell; SDLN, skin draining lymph node; Teff, T effector; Treg, T regulatory.

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.1302401
neuronal survival after injury through regulation of macrophage phenotype.

Materials and Methods

Animals

Female C57BL/6 (stock 000664) and UBC-GFP (stock 004353) mice were purchased from The Jackson Laboratory (Bar Harbor, ME); DEREG mice were a gift of M. Mohrs (Trudeau Institute, Saranac Lake, NY) (27). All animals were housed in temperature- and humidity-controlled rooms, maintained on a 12-h/12-h light/dark cycle (lights on 7:00 A.M.), and age matched in each experiment. All strains were kept in identical housing conditions. All procedures complied with regulations of the Institutional Animal Care and Use Committee at the University of Virginia.

In vivo drug treatment

A total of 200 μg all-trans retinoic acid (ATRA; Fisher) was dissolved in corn oil and injected i.p. every other day starting 3 d before injury. Diphtheria toxin (DTx) was dosed at 40 μg/kg in PBS and was injected into C57BL/6 or DEREG mice 2 d before optic nerve injury and on the day of optic nerve injury. A total of 250 μg anti-CD25 (clone PC-61) was injected into mice 8 d before optic nerve injury.

Retrosgrade labeling of retinal ganglion cells

Mice were anesthetized, and the skull was exposed and immobilized in a stereotactic device. Holes were drilled in the skull above the superior colliculus (bilaterally 2.9 mm caudal to bregma and 0.5 mm lateral to midline). A quantity amounting to 1 μL 4% Fluoro-gold was injected 2 mm below the meningeal surface at a rate of 0.5 μl/min using a Hamilton syringe and an automatic injector. The dye was allowed to diffuse into the tissue for 1 min before the syringe was removed. The scalp was then sutured closed, and the mice were allowed to recover on warming pads at 37°C before returning them to their cage.

Optic nerve injury

Mice were subjected to an optic nerve injury 3 d after stereotactic surgery. Briefly, mice were anesthetized with a 1:1:8 mixture of ketamine:xyazine:saline. An incision was made in the connective tissue above the sclera. The venous sinus around the optic nerve was retracted to expose the optic nerve, the venous sinus around the optic nerve was retracted to expose the optic nerve, and then the SDLN (Fig. 1J–L). To determine whether the immune response to CNS injury was occurring, we first examined CNS-draining dCLN as compared with SDLN. No change in the number or percentage of CD4+ T cells in injured or uninjured deep cervical or skin draining lymph nodes (SDLN) were isolated using magnetic bead separation (Miltenyi Biotech) and incubated at 1 × 10^5 cells/well in complete macrophage media. Twenty-four hours after addition of T cells, the macrophages were washed five times with PBS to remove the nonadherent T cells, and RNA was isolated from the macrophages.

Immunohistochemical staining of optic nerve tissue

For arginase-1 staining, mice were perfused transcardially with ice-cold PBS containing 4 U/ml heparin and then with 4% paraformaldehyde. Eyes were enucleated and frozen on dry ice in OCT. The 10-μm sections were cut on a Lyca cryostat and mounted on gelatin-coated slides. Sections were postfixed in 3:1 acetone:ethanol at 4°C before staining with the following Abs: CD4-FITC (clone GK-1.5), CD11b (BioLegend; clone M1/70), and Foxp3-biotin (eBioscience; clone FJK-16s). For Foxp3 and CD4 co-staining, CD4 was detected with an anti-fluorescein secondary Ab (Life Technologies) and Foxp3 was detected with Alexafluor 594–conjugated streptavidin (Jackson Immunchemical).

Results

A CD4+ T cell response in the CNS-draining dCLN after CNS injury

To determine where the immune response to CNS injury was occurring, we first examined CNS-draining dCLN as compared with SDLN (axillary and inguinal) for T cell activation and proliferation upon CNS injury. We found an increase in the number and percentage of CD4+ T cells and a concurrent reduction in the percentage of CD8+ T cells in CNS-draining dCLN (Fig. 1A–C). No change in the number or percentage of CD4+ T cells was observed in the SDLN (Fig. 1D–F). When the induced CD4+ T cells were examined for subpopulation (Treg versus Thet), both activated Treg (CD4+CD25+Foxp3+) and Thet (CD4+Foxp3-) cells were increased in the dCLN after the injury (Fig. 1G–I), but not in the SDLN (Fig. 1J–L). To determine whether the immune response in the dCLN was playing an important role in the response

For Treg cultures, total lymph nodes were dissected, and a single-cell suspension was made by mashing through a 70-μm mesh. A total of 3 × 10^6 cells/ml was incubated in T cell culture media supplemented with 1 μg/ml anti-CD3 (clone 145-2C11; American Type Culture Collection [ATCC]) and 1 μg/ml anti-CD28 (clone 37.51; Bioxcell, stock BE0015-1). For Treg cells in vitro (iTreg) cultures, the media was supplemented with 10 nM ATRA (Fisher), 5 ng/ml TGF-β (PeproTech), and 250 U/ml IL-2 (R&D Systems). The cultures were maintained for 5 d before CD4+ T cells were isolated using magnetic bead separation (Miltenyi Biotec) and injected i.v. into C57BL/6 mice.

Flow cytometry

Axillary and inguinal lymph nodes or dCLN were isolated and mashed through a 70-μm strainer in PBS containing 1% BSA and 2 mM EDTA. The following Abs were used, and are all from eBioscience, unless otherwise noted: Foxp3-Alexa 488, CD4-PerCP Cy5.5, TCR-β allophycocyanin eFluor780, CD45-allophycocyanin, CD8-eFluor 450, CD25-PE (BD Bioscience), IL-4-PE, IFN-γ allophycocyanin, and CD69-PE Cy7. For intracranial staining, the cells were fixed overnight in Fixp3 Fix/Perm buffer (eBioscience) before incubating with Foxp3 Ab in FACS buffer containing 0.3% saponin (Fisher). For intracellular staining, mice were injected with 3 μg brefeldin A 5 h before they were sacrificed. The cells were stained for extracellular markers before they were fixed in the fixation buffer (eBioscience) for 30 min, then permeabilized and stained for intracellular Ags in FACS buffer containing 0.3% saponin.

Macrophage-skewing assay

Bone marrow was isolated from wild-type mice and cultured on untreated petri dishes in DMEM/F12 containing 10 ng/ml M-CSF (eBioscience), 10% FCS, t-glutamine, and pen-strep (Invitrogen). The media was changed every 3 d, and macrophages were used after 8 d in vitro. The day before the macrophages were used, they were replated on tissue culture–treated 24-well plates. CD4+ T cells from injured or uninjured deep cervical or skin draining lymph nodes (SDLN) were isolated using magnetic bead separation (Miltenyi Biotec) and incubated at 1 × 10^5 cells/well in complete macrophage media. Twenty-four hours after addition of T cells, the macrophages were washed five times with PBS to remove the nonadherent T cells, and RNA was isolated from the macrophages.

Production of bone marrow chimeras

C57BL/6 mice underwent split-dose lethal irradiation (350 rad, then 950 rad 48 h later). Bone marrow cells were isolated by flushing out the femur and tibia of UBC-GFP mice. A total of 1 × 10^7 bone marrow cells was injected into the irradiated mice 3 h after the last irradiation. Mice were allowed to reconstitute for 6 wk before being used in experiments.

T cell cultures

For Treg cultures, total lymph nodes were dissected, and a single-cell suspension was made by mashing through a 70-μm mesh. A total
to CNS injury, we used an optic nerve injury model, in which RGCs are prelabeled with the neuronal tracer Fluoro-gold and then the optic nerve is injured and the number of surviving RGCs in the retina is quantified (Fig. 1M). This injury leads to a decrease in the number of RGCs in mice that underwent the dCLN removal from those that received a sham surgery (Fig. 1N), whereas their contralateral uninjured retinas did not display a loss of RGCs (Fig. 1O).

To determine whether T cells from the injured dCLN displayed a different phenotype after CNS injury than the SDLN, we used flow cytometry to analyze the intracellular cytokines produced in the lymph nodes after injury. T cells from the dCLN displayed higher levels of IL-4 after optic nerve injury than those from the SDLN (Fig. 2A, 2B). To examine whether this phenotype is induced by the injury, we used KN2 reporter mice that express...
human CD2 (hCD2) when IL-4 is being translated. In these mice, hCD2 expression is seen in CD44⁺ memory T cells (Fig. 2C), and this hCD2 expression is induced after injury in the dCLN after injury, but not in the SDLN (Fig. 2D). To determine whether T cells induced after optic nerve injury in the draining lymph nodes are capable of supporting alternative activation of macrophages, we isolated T cells from injured and uninjured dCLN and SDLN and cocultured them with a pure population of bone marrow–derived macrophages. T cells from the dCLN of optic nerve–injured mice were able to support an alternative activation phenotype of bone marrow macrophages in vitro, whereas T cells obtained from SDLN of injured mice or from dCLN of uninjured mice were unable to promote this alternatively activated (tissue-building) phenotype of macrophages (Fig. 2E). This suggests that the injury indeed induces Teff cells in the draining dCLN that are capable of promoting a neuroprotective macrophage phenotype.

Because we observed that T cells in the draining lymph node were able to promote an alternative activation of macrophages, we addressed a possibility that T cells are controlling the phenotype of the infiltrating monocytes/macrophages. Arginase-1–expressing

**FIGURE 2.** CNS injury promotes a milieu conducive to alternative activation of macrophages in the dCLN. (A) Gating strategy and representative staining of IL-4 production by CD4⁺ T cells in the draining dCLN and SDLN after CNS injury. (B) Quantification of the mean fluorescence intensity of IL-4 staining of CD4⁺ T cells in the dCLN or SDLN. n = 4 per group. Representative of two experiments. ***p < 0.001 Student t test. (C) Representative staining of hCD2 (IL-4) production by CD4⁺ T cells in the draining dCLN in injured and uninjured mice. (D) Quantification of the number of hCD2⁺ staining of CD4⁺ T cells in the dCLN or SDLN in uninjured and injured mice. n = 5 per group. Representative of two experiments. *p < 0.05 Student t test. (E) arg1 mRNA expression of bone marrow–derived macrophages that had been cocultured with CD4⁺ T cells from the indicated lymph nodes of mice with or without optic nerve injury for 24 h. n = 3 per group. Representative of >3 experiments. *p < 0.05 one-way ANOVA with Bonferroni’s posttest. (F) Representative images of injured optic nerves of GFP⇒C57BL/6 bone marrow chimeras stained for arginase-1 and Iba1. Arrowheads point to GFP⁺ radio-resistant microglia, whereas arrows point to infiltrating macrophages (scale bars, 100 µm). (G) Quantification of percentage of Iba1⁺ cells in the injured optic nerve that are GFP⁺arginase-1⁺, GFP⁺arginase-1⁻, GFP⁻arginase-1⁺, and GFP⁻arginase-1⁻. n = 3 per group. *p < 0.05 one-way ANOVA with Bonferroni’s posttest. (H) Quantification of the percentage of GFP⁺ and GFP⁻ cells that are arginase-1⁺ in the injured optic nerve. n = 3 per group. ***p < 0.001 Student t test.
macrophages (M2 type) have been previously described to support neuronal survival after CNS injury (24–26, 28). Indeed, using immunohistochemistry of injured optic nerves, we demonstrate that arginase-1 expression is induced in the injury site (Fig. 2F), whereas there is no detectable arginase-1 staining in the uninjured optic nerves (Supplemental Fig. 1A). We established GFP−C57BL/6 bone marrow chimeric mice (29), whose peripheral immune system is replaced by the GFP+ bone marrow, but that have a significant number of GFP− microglia in the optic nerve (Supplemental Fig. 1B). Therefore, despite the issues inherent with bone marrow transplantation following irradiation (30), the large majority of the engrafted Iba1-positive cells in the uninjured CNS of chimeric mice are of non-GFP origin. It is, therefore, conceivable to assume that an increase of GFP+ cells after injury results primarily from their peripheral recruitment of monocyte-derived macrophages, although, as stated above, the procedure has its limitation. In the chimeric mice, most of the GFP−Iba1+ cells in the site of the injury were arginase-1 positive, suggesting that at least the majority of the infiltrating cells were highly skewed after injury. However, significantly fewer of the radio-resistant GFP+Iba1− microglia were arginase-1 positive, suggesting that macrophages infiltrating from the periphery are the primary source of alternatively activated myeloid cells (Fig. 2G, 2H). This preferential skew of infiltrating myeloid cells suggests that their phenotype switch took place in the periphery prior to infiltration rather than in the CNS parenchyma. These results are in line with previous findings, suggesting that monocytes with an alternatively activated phenotype are arriving from a periphery through a unique path into the injured CNS (25).

Depletion of Treg exacerbates neurodegeneration after CNS injury

The contribution of different subsets of T cells to neuronal survival after CNS injury has been intensively studied (4, 5, 19, 31, 32), yet their role in this postinjury neuronal survival remains controversial (8, 10, 33). Because Treg cells are known to exert asymmetric control of T cell responses in nonpathological situations (34), we tested the hypothesis that Treg cells were responding to injury in the draining lymph nodes, where they controlled the phenotype of T eff cells. We used DEREG mice (16), which express the DTx receptor under the Foxp3 promoter to assess the effect of Treg depletion on neuronal survival. Treatment of these mice with 40 µg/kg DTx 2 d before injury completely eliminates Treg cells in the bloodstream (Supplemental Fig. 2A). Seven days after injury, the DEREG mice treated with DTx still displayed decreased numbers of Treg cells in their CNS-draining dCLN (Fig. 3A) and an increase in the number of activated T eff cells in the dCLN and SDLN (Fig. 3B, Supplemental Fig. 2B).

FIGURE 3. Alleviation of Treg suppression after CNS injury leads to a reduced neuronal survival after optic nerve injury. (A and B) Bar graphs represent quantification of flow cytometry analysis of the dCLN of DEREG or wild-type littermates treated with DTx 2 d before injury and on the day of injury, showing percentage of CD25+Foxp3− Treg cells (A) and of CD25+Foxp3+ T eff cells (B), graphed as a percentage of TCR-β+CD4+ cells (n = 12 wild-type and 9 DEREG treated mice). Representative of three experiments. ***p < 0.001, *p < 0.05 Student t test (C) Neuronal survival after optic nerve injury in DEREG and wild-type mice injected with 40 µg/kg DTx 2 d before injury and on the day of injury. Survival is quantified as a percentage of control survival. n = 19 wild type and 25 DEREG. Representative of three experiments. *p < 0.05 Student t test. (D) Quantification of the number of CD4+ T cells found in the injury site of DEREG mice treated with DTx normalized to the number of CD4+ T cells found in the injury site of C57BL/6 mice treated with DTx. n = 3 per group. Representative of two experiments. Student t test. (E) Quantification of the number of CD11b+ cells found in the injury site, normalized to the number of CD11b+ T cells found in the injury site of C57BL/6 mice treated with DTx. n = 3 C57BL/6 treated with DTx and 9 DEREG treated with DTx. Representative of two experiments. Student t test. (F) Representative images of C68 and arginase-1 in injured optic nerve of DEREG and wild-type mice treated with two doses of 40 µg/kg DTx. Scale bars, 100 µm. (G) Arginase-1− area graphed as a percentage of CD68− area in C57BL/6 or DEREG mice treated with DTx. n = 3 C57BL/6 treated with DTx and 8 DEREG treated with DTx. *p < 0.05 Student t test. (H) Quantitative PCR for arg1 of optic nerves of C57BL/6 or DEREG mice treated with 40 µg/kg DTx 2 d before injury and on the day of injury normalized to arg1 expression in the contralateral uninjured nerve. n = 7 C57BL/6 treated with DTx and 4 DEREG treated with DTx. Representative of two experiments. *p < 0.05 Student t test.
To test the effect of Treg cell depletion on CNS injury, we again used the optic nerve crush injury model. As expected from previous studies (35), DEREG mice treated with DTx, and thus depleted of Treg cells, showed a decrease in the number of surviving RGCs 7 d after injury, as compared with wild-type mice treated with DTx (Fig. 3C). We examined the contralateral retina of injured mice (Supplemental Fig. 2C) and histological sections of uninjured mice treated with DTx (Supplemental Fig. 2D), which did not display any loss of RGCs or immune cell infiltrate, suggesting DTx by itself did not have destructive effects on uninjured CNS tissue. Furthermore, there was no difference in neuronal survival in C57BL/6 mice treated with saline or DTx (Supplemental Fig. 2E), confirming that DTx treatment was not causing nonspecific effects at the dose that we are using. To further establish the role of depletion of Treg cells, we used an anti-CD25 Ab, which depletes Treg cells (that express high levels of CD25) (36). In mice that were Treg depleted, there was a decrease in the number of CD25+Foxp3+ Treg cells in the dCLN even 7 d after optic nerve injury and a corresponding decrease in neuronal survival (Supplemental Fig. 2F, 2G), consistent with the results seen in DEREG mice.

Although no change in overall numbers of CD4+ T cells (Fig. 3D) or CD11b+ myeloid cells (Fig. 3E) at the site of injury was found, the percentage of accumulated macrophages was altered in DEREG mice treated with DTx. A significant decrease in arginase-1–expressing CD68 (a marker of activated macrophages) cells was evident (Fig. 3F, 3G), suggesting a decrease in alternatively activated macrophages (M2 type) after injury in Treg-depleted mice. No difference in the total amount of CD68+ area was detected (Supplemental Fig. 3A). To confirm the histological observations, we also examined the injured tissue by PCR.

The mRNA expression of arg1, the gene for arginase-1, was reduced in DEREG mice, confirming the decrease in alternatively activated macrophages after Treg depletion (Fig. 3H).

**Exogenous Treg cells inhibit a beneficial response to CNS injury**

A complete depletion of Treg cells using DEREG mice resulted in impaired outcome of CNS injury in our optic nerve crush injury model (Fig. 3C). However, the question still remains whether increased activity of Treg cells would conversely offer a benefit after CNS injury. First, we tested the physiological outcome of Treg manipulation via potentiation of Treg-suppressive function by treating mice with ATRA, which induces differentiation of Treg cells (37), stabilizes the Treg phenotype (38), and makes Treg cells more suppressive (38). As expected, treatment of mice with ATRA increased the Treg population in the dCLN after injury (Fig. 4A), but surprisingly not in the SDLN (Supplemental Fig. 4A), and resulted in a decrease of activated Teff cells in the dCLN and SDLN (Fig. 4B, Supplemental Fig. 4B). Interestingly, and in line with some reports (19) but contrary to other previous findings (7), mice treated with ATRA exhibited decreased neuronal survival compared with vehicle-treated mice (Fig. 4C), suggesting that induction of highly suppressive Treg cells limits the protective Teff responses. To rule out a possible in vivo effect of ATRA on cells other than T cells, we differentiated iTreg using ATRA and TGFβ (Supplemental Fig. 4C). Injection of iTreg cells into CNS-injured mice also resulted in an increase in Treg cells (Fig. 4D) and attenuation of their activated Teff response to injury in the dCLN (Fig. 4E), but no change in the number of Treg cells and Teff cells in the SDLN (data not shown) and a reduction in neuronal survival (Fig. 4F). Mice treated with Teff cells (that were activated without TGF-β and ATRA, and that contained only ∼3% Foxp3+ Treg

**FIGURE 4.** Potentiation of Treg function impairs neuronal survival after optic nerve injury. (A and B) Bar graphs represent quantification of flow cytometry analysis of the dCLN of wild-type mice treated with vehicle or ATRA showing percentage of CD25+Foxp3+ Treg cells (A) and of CD25+Foxp3− Treg cells (B), graphed as a percentage of TCR-β+CD4+ cells. n = 7 vehicle treated and n = 9 ATRA treated. Representative of two experiments. *p < 0.05 Student t test. (C) RGC survival in wild-type mice treated with vehicle or ATRA. Survival is quantified as a percentage of control survival. n = 7 vehicle and n = 9 ATRA. Representative of two experiments. *p < 0.05 Student t test. (D and E) Bar graphs represent quantification of flow cytometry analysis of the dCLN of wild-type mice treated with vehicle or 1 × 10^6 exogenous Treg cells 1 d before injury and 1 d after injury, showing percentage of CD25+Foxp3+ Treg cells (D) and of CD25+Foxp3− Treg cells (E), graphed as a percentage of TCR-β+CD4+ cells. n = 12 Treg cells injected and n = 13 vehicle injected. *p < 0.05 Student t test. (F) Neuronal survival in wild-type mice injected with vehicle, 1 × 10^6 Treg or 1 × 10^6 Teff cells 2 d before injury and on the day of injury. Survival is quantified as a percentage of control survival. n = 13 vehicle injected, 12 Treg cell injected, and 7 Teff cell injected. Representative of two experiments. *p < 0.05 one-way ANOVA with Bonferroni’s posttest.
compared with ~85% in Treg-designated culture conditions) did not show any change in neuronal survival (Fig. 4F), possibly due to the large number of Teff cells already present in wild-type mice.

Interestingly, 7 d after Treg injection, only very few of the injected cells were found in the dCLN, despite a substantial increase in overall Treg numbers (Supplemental Fig. 4D). These results suggest that the injected Treg cells induce endogenous T cell differentiation, possibly through their high levels of secreted and membrane-bound TGF-β (39, 40).

Treg cells do not infiltrate the injured CNS after injury

Because Treg cells are exerting a negative effect on the outcome to CNS injury, we sought to determine whether Treg cells were also gaining access to the site of injury. Upon injury, there is the influx of Teff cells to the CNS parenchyma (Fig. 5A). Despite being able to visualize Treg cells using Foxp3 immunolabeling in the spleen (Fig. 5B) and in spinal cords that had been injected directly with Treg cells (Fig. 5C), we did not see Treg cells in the parenchyma of the injured optic nerve in animals after exogenous i.v. injection of Treg cells (Fig. 5D) or in injured wild-type mice, wild-type mice injected with Teff cells, DEREG mice treated with DTx, and wild-type mice treated with DTx (data not shown). Furthermore, there was no difference in the number of CD4+ Teff cells at the injury site of Treg-treated mice (Fig. 5E). Therefore, it seems unlikely that acute manipulation of Treg cells in our experimental paradigms is affecting neuronal survival through the migration of Teff cells into the site of the injury. Next, to determine whether addition of Treg cells could affect monocyte migration to the injured CNS, we quantified the number of CD11b+ cells accumulating at the site of injury. As with CD4+ T cells, there was no change in the number of CD11b+ cells that migrated to the site of injury in Treg-treated mice (Fig. 5F, 5G), further suggesting that Treg cells are not controlling immune cell migration in this injury model.

To determine whether T cell–derived cytokines are affected by Treg manipulation after CNS injury, we examined the mRNA expression of genes from the optic nerve of injured mice treated with either Teff or Treg cells. Mice treated with Treg cells display a dramatic decrease in the amount of IL-4 mRNA compared with mice treated with Teff cells (Fig. 6A), suggesting a change in the Th2 response to damage at the injury site with Treg treatment. To determine whether these changes are having effects downstream on myeloid cells after CNS injury, we examined markers of myeloid skewing in the injured optic nerves of mice treated with Treg cells. Indeed, Treg-treated mice displayed a decrease in the mRNA expression of alternatively activated macrophage markers arg1 and il10, whereas there was no change in the classical activation markers nos2 and tnf (Fig. 6B–E). To further demonstrate that there was a loss of alternative activation of macrophages in Treg-injected mice, we examined colocalization between CD68, a marker of activated myeloid cells, and arginase-1 by immunofluorescence. Although Teff-injected mice displayed marked expression of arginase-1 in the CD68+ fraction 7 d after injury, injection of Treg cells led to a decrease in arginase-1 expression by myeloid cells (Fig. 6F, 6G), further demonstrating that both addition and deletion of Treg cells are detrimental in CNS trauma through their effects on the innate immune response at the site of injury.
**Discussion**

There has been an ongoing debate about the role of Treg cells in CNS injury and in neurodegenerative conditions. We show in this work that complete depletion of Treg cells using DEREG mice, a manipulation that has been shown to lead to development of numerous organ-specific autoimmune diseases (16), leads to increased neurodegeneration after CNS trauma. However, the same deleterious effect on neuronal survival can be seen when Treg numbers are increased either by pharmacological compounds or by exogenous supply of Treg cells. Changes in Treg numbers correlate with the phenotype of macrophages populating the injury site, with alternatively activated macrophages being spontaneously induced by the injury, yet inhibited by Treg cell addition or overruled by an exaggerated immune response as a result of Treg cell depletion.

Early work in models of stroke and Parkinson’s disease showed that depletion of Treg cells led to increased neurodegeneration, whereas increases in Treg cell numbers and function improved disease outcome (10, 41). More recent work, using the same manipulations, has shown that Treg cells play a detrimental role after CNS injuries (7), supporting the hypothesis that they are suppressing a beneficial autoimmune response (19). There are several factors that may have contributed to these disparate findings. There are technical challenges with the current Treg depletion strategies that have hindered interpretation of depletion studies, such as targeting of activated effector cells with anti-CD25 treatment (42, 43). Furthermore, several studies have shown that Treg cells have the potential to downregulate Foxp3 and become effector cells, especially when placed in lymphopenic or inflammatory conditions (38, 44, 45), probably due to heterogeneity in the fate commitment of the Treg cell population (46), complicating transfer experiments into mice with abnormal adaptive immune systems. However, the conditions that drive this switch from Treg to Teff, and relevance of these models in vivo, is still a matter of debate (47). Although DTx treatment may have indirect effects, overall the DEREG mouse offers a unique model for Treg depletion in vivo by both avoiding targeting CD25, indirectly affecting activated Teff, and avoiding cell transfer, which inevitably changes the cell phenotype. Therefore, although imperfect, DEREG model presents the best currently available model for Treg depletion.

Our work further addresses the question of how Treg cells are affecting the outcome from CNS injury. Although we show that Treg cells have profound effects on neuronal survival from injury, they are not found at the site of the injury, but are rather enriched in the draining lymph node. Treg cells are known to exert asymmetric control of T cell responses in nonpathological situations (34), raising the possibility that these Treg cells are exerting their action on the phenotype of Teff cells in the draining lymph node. The Teff cells, in turn, direct the phenotype of the infiltrating innate immune cells. Previous works demonstrated that precursors for alternatively activated macrophages are recruited to the injured CNS through a unique path of the choroid plexus (25) and are coming predetermined to differentiate into alternatively activated macrophages.

Several studies have shown that alternative activation of macrophages is a beneficial response to CNS injury (25, 28). Tissue-building macrophages produce growth factors, such as insulin-like growth factor 1, vascular endothelial growth factor, TNF-β, and factors that remodel the extracellular matrix, such as matrix metalloproteases and resistin-like molecule alpha and promote a tissue-building phenotype in injured tissue (21). T cells, and specifically Th2 effector cells, produce several cytokines, such as IL-4 for example, that can induce alternatively activated macrophages (48). Our results suggest that T cells induced in the CNS-draining dCLN control the phenotype of the infiltrating monocytes, and future studies need to concentrate on better understanding the molecular interaction between T cells and myeloid cells that results in myeloid cells of a particular phenotype to migrate to the site of injury.
injury. There potentially could be additional mediators between T cell response and the phenotype of macrophages recruited to the site of injury. It is also unknown whether T cells recirculate to the injured CNS site and the draining dCLN. If such a recirculation occurs, T cell–macrophage interaction could potentially take place in three different sites—the site of injury, the lymph nodes, and blood. Although we show in this work that removal of dCLN is detrimental for neuronal survival, we have not addressed whether their removal after the injury has been inflicted could affect the phenotype of macrophages and, subsequently, neuronal survival.

Our results support the notion that a spontaneous immune response after CNS trauma is beneficial and is tightly regulated by Treg cells (19). Elimination of Treg leads to an excessive immune response that is detrimental for injured tissue. However, injection of Treg cells or potentiation of their suppressive function inhibits a spontaneous immune response to injury and also results in impaired neuronal survival. Further works should be aimed at understanding the divergent properties of Treg cells that lead to this dichotomous response to injury and finding the compounds that could alleviate Treg function, yet preserve the beneficial nature/phenotype of Treg cells. Without better understanding of Treg interactions after CNS injury, therapies for CNS injuries that primarily target the Treg compartment should be taken with extreme caution, as alteration of Treg may result in impaired outcome of CNS trauma.

Acknowledgments

We thank Shirley Smith for editing the manuscript. We thank the members of this work.

References


**Supplementary Figure 1:** Uninjured optic nerves have few GFP⁺Iba₁⁺ microglia and little arginase-1, but arginase-1 staining is greatly increased after injury. (a) Immunohistochemical staining of arginase-1 (green) and CD68 (red) in the uninjured and injured optic nerve (scale bar = 100 µm). (b) Immunohistochemical staining of an optic nerve in a C57Bl/6 mouse that was lethally irradiated and transplanted with GFP bone marrow showing sparse transplanted GFP⁺ cells (green) among plentiful resident Iba₁⁺ microglia (red). Image is from uninjured nerve 6 weeks post-transplantation (scale bar = 100 µm).
Supplementary Figure 2: Characterization of 40 µg/kg DTx treatment. (a) Flow cytometry on the day of injury of CD4 and FoxP3 in peripheral blood of DEREG mice treated with vehicle or 40 µg/kg DTx. (b) Flow cytometry in the skin-draining lymph nodes of DEREG or wild type littermates treated with DTx two days before injury and on
the day of injury, showing percent of CD25^+Foxp3^- T_{eff} cells, graphed as a percentage of TCR\^\beta^-CD4^+ cells (n = 12 wild type and 9 DEREG treated mice; *, p < 0.05, Student’s t-test; representative of three experiments). (c) Representative images of H&E staining in eyes of DEREG mice treated with 40 \mu g/kg DTx. Abnormal architecture or immune cell infiltration was found in 0/17 C57Bl/6 and 0/21 B6AF1 mice. (d) Retinal ganglion cell counts from DEREG and wild type mice injected with 40 \mu g/kg DTx two days before injury and on the day of injury (n = 19 wild type and 25 DEREG; Student’s t-test; representative of three experiments). (e) No difference was observed in RGC survival of C57Bl/6 mice treated with 40 \mu g/kg DTx or saline 2 days before injury and on the day of injury (n = 10 mice per group; Student’s t-test; representative of 2 experiments). (f) Flow cytometry in the skin-draining lymph nodes of wild type mice treated with 250\mu g anti-CD25 or IgG 8 days before injury, showing percent of CD25^+Foxp3^+ T_{reg} cells, graphed as a percentage of TCR\^\beta^-CD4^+ cells (n = 4 per group; *, p < 0.05, Student’s t-test; representative of three experiments). (g) RGC survival of C57Bl/6 mice treated with 250 \mu g anti-CD25 or IgG 8 days before injury (n = 6 IgG and 7 anti-CD25; *, p < 0.05, Student’s t-test; representative of 2 experiments).
Supplementary Figure 3: There is no change in CD68$^+$ area after injury with $T_{reg}$ manipulation. (a) Quantification of CD68$^+$ area in immunohistochemical staining of injured optic nerves of C57Bl/6 treated with DTx or DEREG mice treated with DTx (n = 3 C57Bl/6 treated with DTx and 9 DEREG treated with DTx Student’s t-test; representative of two experiments). (b) Quantification of CD68$^+$ area in immunohistochemical staining of injured optic nerves of C57Bl/6 mice injected with 1x10^6 $T_{eff}$ or $T_{reg}$ cells 2 days before injury and on the day of injury (n = 9 $T_{eff}$ injected and 6 $T_{reg}$ injected; Student’s t-test; representative of two experiments).
Supplementary Figure 4: ATRA treatment leads to decreased T_{eff} proportion in the skin-draining lymph nodes and to increased T_{reg} proportion in vitro. (a, b) Flow cytometry in the skin-draining lymph nodes of wild type treated with vehicle or ATRA showing percent of CD25^{+}Foxp3^{+} T_{reg} cells (a) and of CD25^{+}Foxp3^{−} T_{eff} cells (b), graphed as a percentage of TCR\beta^{+}CD4^{+} cells (n = 7 vehicle treated and n = 9 ATRA treated; *, p <
0.05, Student’s t-test; representative of two experiments). (c) Representative flow cytometry plots of viable cells in lymph node cultures that have been treated with 10 nM ATRA, 5 ng/mL TGFβ, 250 U/mL IL-2, 1 µg/mL anti-CD3, and 1 µg/mL anti-CD28 (T_{reg}) or only 1 µg/mL anti-CD3, and 1 µg/mL anti-CD28 (T_{eff}) for 5 days. The boxes denote the CD4^{+}Foxp3^{+} T_{reg} population, and numbers represent percent of viable cells that are CD4^{+}Foxp3^{+}. (d) Representative flow cytometry plots of viable cells in the deep cervical lymph node cultures after injection of 1 X 10^6 Thy1.1 T_{reg} cells or vehicle. The gates on the top plots denote the CD4^{+}Foxp3^{+} T_{reg} cells, and the gates on the bottom denote the Thy1.1^{+} fraction of T_{reg} cells.