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Pathological CNS Autoimmune Disease Triggered by Traumatic Spinal Cord Injury: Implications for Autoimmune Vaccine Therapy

T. Bucky Jones,[1] D. Michele Basso,[1,2] Ajeet Sodhi,[5] Jonathan Z. Pan,[5] Ronald P. Hart,[5,6] Robert C. MacCallum,[3] Sunhee Lee,[3] Caroline C. Whitacre,[1,4] and Phillip G. Popovich[1,4]

[1]The Neuroscience Graduate Studies Program, [2]Division of Physical Therapy, School of Allied Medical Professions, [3]Department of Psychology, College of Social and Behavioral Sciences, and [4]Department of Molecular Virology, Immunology and Medical Genetics, The Ohio State University College of Medicine and Public Health, Columbus, Ohio 43210, [5]Department of Biological Sciences, New Jersey Institute of Technology and Rutgers University, Newark, New Jersey 07102, and [6]W. M. Keck Center for Collaborative Neuroscience, Rutgers University, Piscataway, New Jersey 08854

Lymphocytes respond to myelin proteins after spinal cord injury (SCI) and may contribute to post-traumatic secondary degeneration. However, there is increasing evidence that autoreactive T-lymphocytes may also convey neuroprotection and promote functional recovery after CNS injury. To clarify the role of myelin autoreactive lymphocytes after SCI, we performed contusion injuries in the thoracic spinal cord of transgenic (Tg) mice in which >95% of all CD4+ T-lymphocytes are reactive with myelin basic protein (MBP). We observed significantly impaired recovery of locomotor and reflex function in Tg mice compared with non-Tg (nTg) littermates. Measures of functional impairment in Tg mice correlated with significantly less white matter at the injury site, and morphometric comparisons of injured Tg and nTg spinal cords revealed increased rostrocaudal lesion expansion (i.e., secondary degeneration) in Tg mice. Rostrocaudal to the impact site in SCI–nTg mice, demyelination was restricted to the dorsal funiculus, i.e., axons undergoing Wallerian degeneration. The remaining white matter appeared normal. In contrast, lymphocytes were colocalized with regions of demyelination and axon loss throughout the white matter of SCI–Tg mice. Impaired neurological function and exacerbated neuropathology in SCI–Tg mice were associated with increased intraspinal production of proinflammatory cytokine mRNA; neurotrophin mRNA was not elevated. These data suggest that endogenous MBP-reactive lymphocytes, activated by traumatic SCI, can contribute to tissue injury and impair functional recovery. Any neuroprotection afforded by myelin-reactive T-cells is likely to be an indirect effect mediated by other non-CNS-reactive lymphocytes. Similar to the Tg mice in this study, a subset of humans that are genetically predisposed to autoimmune diseases of the CNS may be adversely affected by vaccine therapies designed to boost autoreactive lymphocyte responses after CNS trauma. Consequently, the safe implementation of such therapies requires that future studies define the mechanisms that control T-cell function within the injured CNS.

Key words: protective autoimmunity; neuroprotection; CNS injury; spinal cord injury; myelin basic protein; autoimmune disease

The cellular inflammatory response induced by spinal trauma is comprised of resident glia (microglia and astrocytes), infiltrating neutrophils, monocytes, and lymphocytes reactive with a variety of antigens, including myelin basic protein (MBP) (Dusart and Schwab, 1994; Popovich et al., 1996, 1997). After CNS injury, T-lymphocytes (T-cells), including those reactive with MBP, accumulate at the injury site (Hirschberg et al., 1998). The biological impact of this T-cell infiltration remains controversial. Previously, we and others demonstrated that MBP-reactive T-cells are activated after spinal contusion injury (SCI) in rats and humans (Popovich et al., 1996; Kil et al., 1999) and that these cells are capable of inducing paralytic disease and neuropathology reminiscent of the T-cell-mediated autoimmune disease, experimental autoimmune encephalomyelitis (EAE) (Popovich et al., 1996). However, exogenous administration or *in vivo* expansion of CNS autoreactive cells via vaccination is being proposed as a potential clinical therapy for a variety of neurodegenerative conditions, including SCI (Cohen and Schwartz, 1999; Schwartz et al., 1999a; Hauben et al., 2000b), Alzheimer's disease (Morgan et al., 2000), and glaucoma (Fisher et al., 2001). After spinal contusion trauma in rats, injection of MBP-reactive T-cell lines or immunization with MBP causes significant neuroprotection and functional recovery (Hauben et al., 2000a). The mechanisms underlying this neuroprotection have not been defined but could be the result of activated lymphocytes that accumulate at the injury site (as a result of SCI or the immunization protocol) that are not reactive to CNS proteins. In fact, non-neuroantigen-specific T-cells represent >90% of the lymphocyte infiltrate in models of T-cell-mediated autoimmune disease (Cross et al., 1990) and have been shown to ameliorate the paralytic disease and histopathology associated with EAE (Falcone and Bloom, 1997). Because these cells could explain the neuroprotection afforded to SCI rats receiving MBP-reactive T-cells (Hauben et al., 2000a), we evaluated the neurological and pathological sequelae of SCI in T-cell receptor (TCR) transgenic (Tg) mice in which the majority of the lymphocyte repertoire (>95% of all

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Correspondence should be addressed to Dr. Phillip Popovich, Department of Molecular Virology, Immunology and Medical Genetics, 2078 Graves Hall, 333 West 10th Avenue, Columbus, OH 43210. E-mail: Popovich.2@osu.edu.

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Table 1. PCR primers (all sequences are 5′ to 3′)

Cytokine/neurotrophin	Forward primer	Reverse primer
IFN-γ	AGCTCATCCGAGTGGTCCAC	GCTTCCTGAGGCTGGATTCC
IL-2	GTCAACAGCGCACCCACTT	TGCTTCCGCTGTAGAGCTTG
IL-1β	CCAAAAGATGAAGGGCTGCT	TCATCAGGACAGCCCAGGTC
IL-4	CACGGATGCGACAAAAATCA	CTCGTTCAAAATGCCGATGA
IL-5	ACAGACATGCACCATTGCCA	TGGGTTCCATCTCCAGCACT
IL-6	TTCCATCCAGTTGCCTTCTTG	GAAGGCCGTGGTTGTCACC
TNFα	ATGCTGGGACAGTGACCTGG	CCTTGATGGTGGTGCATGAG
IL-10	CCTGGTAGAAGTGATGCCCC	TCCTTGATTTCTGGGCCATG
IL-12	GGATGGAAGAGTCCCCCAAA	CTGGAAAAAGCCAACCAAGC
NT-3	TCCTCAGCCATTGACATTCG	GATCTCCCCCAGCACTGTGA
BDNF	AGGCACTGGAACTCGCAATG	AAGGGCCCGAACATACGATT

CD4+ T-cells) is reactive with the immunodominant epitope of MBP (Lafaille et al., 1994). The minimal contribution by non-neuroantigen-reactive T-cells in this model allowed us to determine whether CNS autoreactive lymphocytes are sufficient to exert functionally significant neuroprotection after SCI.

MATERIALS AND METHODS

Transgenic mice. MBP TCR Tg mice were generated by breeding Vα4/Vβ8.2 MBP TCR Tg mice (nucleus of the breeding colony provided by Dr. Charles Janeway, Yale University, New Haven, CT) with B10.PL mice (Jackson Laboratory, Bar Harbor, ME). Progeny were screened for expression of the Vβ8.2 transgene (>95% CD4+ T-cells) by flow cytometry of peripheral blood. Uninjured Tg mice housed in a conventional animal facility with HEPA-filtered air showed no indication of spontaneous autoimmune disease (n = 10 evaluated up to 5 months).

Spinal cord injury. In three independent experiments, 16 Tg and 16 nTg (littermate) mice received a spinal contusion injury as described previously (Jakeman et al., 2000). Briefly, mice were anesthetized [ketamine (80 mg/kg, i.p.) and xylazine (40 mg/kg, i.p.)] and treated with prophylactic antibiotics (Gentocin; 1 mg/kg, s.c.). We performed a laminectomy at vertebral level T9/10 and displaced the exposed dorsal surface of the spinal cord a calibrated vertical distance (0.8 mm) using an electromagnetic SCI device. Aseptic conditions were maintained throughout the procedure. After the injury, manual bladder expression was performed three times daily, and prophylactic antibiotic treatment (Gentocin; 50 mg/d) was given throughout the study to eliminate infectious complications. All animals were housed in Bio-Clean units with HEPA-filtered air. Animals receiving spinal contusions in which the biomechanical variables of the injury (either force, impulse, or momentum of the hit) exceeded 3 SDs were excluded from the study (n = 1).

Behavioral analyses. Beginning 2 weeks before surgery, animals (n = 10/group) were acclimated to the environment in which behavioral evaluation was performed (6 sessions of 15 min each). We assessed recovery of motor function using a standardized open-field locomotor rating scale based on operational definitions of hindlimb movement, paw placement, and coordination (Basso et al., 1995). This test was performed with observers blinded to the treatment group and has been shown to produce consistent results with high inter-rater reliability (Basso et al., 1995, 1996). At 1, 3, 7, 10, and 14 d after injury (dpi) and weekly thereafter, animals were placed into the testing environment and scored over a period of 4 min. Within a group, individual hindlimb scores were averaged and presented as a function of time after injury. Propriospinal (spinal reflex) (Goldberger et al., 1990) and vestibulospinal reflexes (Magnus, 1926; Pellis et al., 1991) were tested in 6 uninjured littermate controls and in four injured Tg (SCI–Tg) and nTg (SCI–nTg) mice (total of 14 mice). All reflexes were analyzed from videotape, field-by-field (60 fields/sec), by a rater blinded to group assignment. With the animal's vision occluded, we tested proprioceptive placing by displacing the left ankle against a 2-mm-thick platform that normally elicits flexion of the hindlimb, allowing the animal's limb to clear the surface, followed by extension until the paw is placed on the platform. We recorded the number of trials out of 10 in which a full flexion–extension reflex, flexion-only, or no hindlimb response was elicited. For air righting (dependent on intact vestibulospinal pathway), mice were released from a supine position 28.5 cm above a foam pad. Normally, the forequarters rotate to prone followed by the hindquarters enabling the mouse to land prone. We recorded the percentage of trials (four total trials) in which a supine landing occurred for each mouse.

Tissue processing. To assess SCI-associated changes in peripheral cytokine production and intraspinal cytokine-neurotrophin mRNA, the spleen and spinal cord were removed from all animals at days 0, 3 (peripheral cytokines only), 7, and 21 after injury. Briefly, animals were anesthetized (see above), the blood supply to the spleen was clamped, and animals were perfused intracardially with sterile PBS (0.1 M, pH 7.4). Spleens were processed for ELISPOT analysis (see below). Spinal cords were rapidly removed and snap-frozen in 2-methylbutane cooled with liquid nitrogen. A 4 mm segment of spinal cord centered on the impact site was removed for extraction of RNA. For histological and immunohistological analyses (10 weeks after injury), animals were anesthetized then perfused intracardially with PBS (0.1 M, pH 7.4) followed by 4% paraformaldehyde in 0.1 M PBS. Spinal cords were removed and processed for histological analyses as described previously (Popovich et al., 1997).

Histological and immunohistological analyses. Serial sections were cut on a cryostat (14 μm) then thaw mounted onto Superfrost+ slides (Fisher Scientific, Pittsburgh, PA). Myelin was evaluated using luxol fast blue (LFB) histochemistry. T-lymphocytes, axons, and fibronectin (to identify connective tissue matrix at the impact site) were evaluated in injured tissues using monoclonal or polyclonal antibodies. Nonspecific antibody binding was minimized by incubating tissue sections with 4% mouse serum in PBS. Polyclonal anti-neurofilament (1:8000; Chemicon, Temecula, CA), monoclonal anti-CD3 (to label lymphocytes; 1:500; Serotec, Raleigh, NC), or polyclonal anti-fibronectin (1:500; Sigma, St. Louis, MO) was applied to tissue sections overnight at 4°C. Sections were rinsed three times in PBS then incubated with biotinylated secondary antibody (1:800) for 1 hr at room temperature. Endogenous peroxidase activity was quenched by incubating tissue sections in 6% H_2O_2 diluted in methanol after which Elite-ABC reagent (Vector Laboratories, Burlingame, CA) was applied to tissues for 1 hr at room temperature. DAB substrate was used to visualize bound antibody.

Spinal cord cytokine and neurotrophin mRNA analysis. We assessed cytokine and neurotrophin mRNA in injured (7 and 21 dpi) and uninjured spinal cord (n = 3–4/group per time point) by quantitative reverse transcription PCR (Q-RT-PCR) using selected gene-specific primer pairs (Table 1). Briefly, total RNA was purified from a 4 mm segment of spinal cord centered on the impact site (or at T9/10 for uninjured tissue) using Trizol (Invitrogen, Carlsbad, CA) followed by RNeasy (Qiagen, Valencia, CA) binding, and quantified by spectrophotometry. cDNA was prepared from RNA by reverse transcription with SuperScript II and random primers as suggested by the manufacturer (Invitrogen). The PCR reactions were performed using 10 ng of cDNA, 50 nM of each primer, and SYBR Green master mix (Applied Biosystems) in 20 μl reactions. Levels of Q-RT-PCR product were measured using SYBR Green fluorescence collected during real-time PCR on an Applied Biosystems 7900HT system (Ririe et al., 1997). Standard curves were generated for each gene using a control cDNA dilution series. Melting point analyses were performed for each reaction to confirm single amplified products.

Peripheral cytokine analysis. Single cell suspensions were prepared from spleens of uninjured and spinal injured Tg and nTg mice at 3, 7, and 21 dpi (n = 3–5/group per time point): time points known to correspond

with SCI-induced changes in phenotypic and functional activation of peripheral lymphocytes (Popovich et al., 1996, 2001). We assessed lymphocyte cytokine production from splenocytes in response to MBP stimulation using ELISPOT analysis. Briefly, plates were coated with capture antibody and incubated overnight at 4°C. Antibodies used include: anti-interleukin (IL)-2 (2 μg/ml); anti-IL-4 (4 μg/ml); anti-IL-5 (5 μg/ml); anti-interferon (IFN)-γ (4 μg/ml); (PharMingen, San Diego, CA); and chicken anti-TGF-β (4 μg/ml) (R&D Systems, Minneapolis, MN). Cells were plated in duplicate (0.5×10^6 cells/well) with 40 μg/ml guinea pig MBP, 40 μg/ml ovalbumin (Sigma), 2 μg/ml anti-CD3ε (IL-2, IFN-γ, IL-4, and IL-5; PharMingen) or 2 μg/ml anti-IgM; (TGF-β; R&D Systems) as a positive control. After incubations of 24 (IL-2, IFN-γ) or 48 (IL-4, IL-5, TGF-β) hr, biotinylated secondary antibodies conjugated to alkaline phosphatase were added, and plates were reacted with 5-bromo-4-chlor-indolyl-phosphate/nitroblue-tetrazolium-chloride phosphatase substrate (Kirkegaard & Perry Laboratories, Washington, DC) to visualize cytokine production. The frequency of cytokine-secreting cells per well was quantified using a KS ELISPOT image analysis system (Zeiss, Oberkochen, Germany) (Benson et al., 1999). To control for interassay variability, values obtained in media wells were subtracted from those obtained from MBP-stimulated wells. IL-10 production from antigen-stimulated T-cells was quantified using commercially available mouse-IL-10 ELISA kits (PharMingen).

Image analysis. Morphometric analyses were performed using computer-assisted image analysis (MCID 5+; Imaging Research Inc., St. Catherines, Ontario, Canada). Briefly, the impact site and sections located 100 μm rostral and caudal, were used for analysis of myelin sparing. This analysis is a reliable anatomical predictor of motor recovery (Kuhn and Wrathall, 1998; Jakeman et al., 2000). Values represent the mean of three adjacent sections analyzed per animal at the impact site (n = 4/group). Tissue areas in which normal spinal cord architecture was absent and/or demyelination or fibrosis was present were defined as lesion. These areas were outlined manually from digitized images and expressed as a percentage of the total spinal cord cross-sectional area at the level being analyzed. Tissue sections were analyzed over a distance of ~7 mm. Representative animals were used for three-dimensional reconstruction of the lesion (M3D package; Imaging Research Inc.). Because of the anisotropic arrangement of neuritic sprouts and/or regenerating axons, meaningful axon counts could not be obtained in a single plane of section. Instead, the area occupied by neurofilament-positive (NF+) axons was quantified using image analysis within the lesion center (0.2 mm^2 sample area). Using this technique, quantitatively larger NF+ areas would reflect increased numbers of axons, larger caliber axons, and/or increased branching of a given axon.

Statistical analyses. To determine whether it was appropriate to combine locomotor recovery data from two separate experiments, ANOVA was conducted with one within-subjects factor (day of measurement) and one between-subjects factor (experiment). The dependent variable was the Basso–Beattie–Bresnahan (BBB) score. Results showed no significant overall mean difference in recovery between experiments ($F_{(1,16)}$ = 0.43; p = 0.52) and no significant day × experiment interaction ($F_{(1,16)}$ = 0.24; p = 0.63). Accordingly, BBB scores from the two experiments were combined for further analyses.

Nonlinear mixed models were applied to determine if the initial rate of recovery and overall functional outcome were different between SCI–Tg and SCI–nTg mice (Crowder and Hand, 1990; Goldstein, 1995). Using this approach, the pattern of change for each animal was assumed to follow a specified functional form, with that function having parameters that have specific meaning (e.g., initial level, asymptote). Each parameter in the function may be defined as either constant across individuals (fixed) or varying across individuals (random). Furthermore, the variation in random parameters may be modeled as a function of characteristics of the individual animals (e.g., genetic makeup, experimental treatment). The following three-parameter nonlinear function was specified to approximate the observed pattern of change seen in Figure 1*A*.

$$Y = a - (a - b)\exp^{-(d-1)c} + e \quad (1)$$

Here Y represents the BBB index, the measure of recovery, a is asymptote, b is initial level, c is initial rate of change, d is day, and e is the residual error. We defined initial level as fixed at 0 for every animal because almost every animal exhibited a BBB score of 0 at day 1 in our data. We allowed initial rate and asymptote to vary across animals, and we used group to predict that variation. Group 1 was coded as a dummy

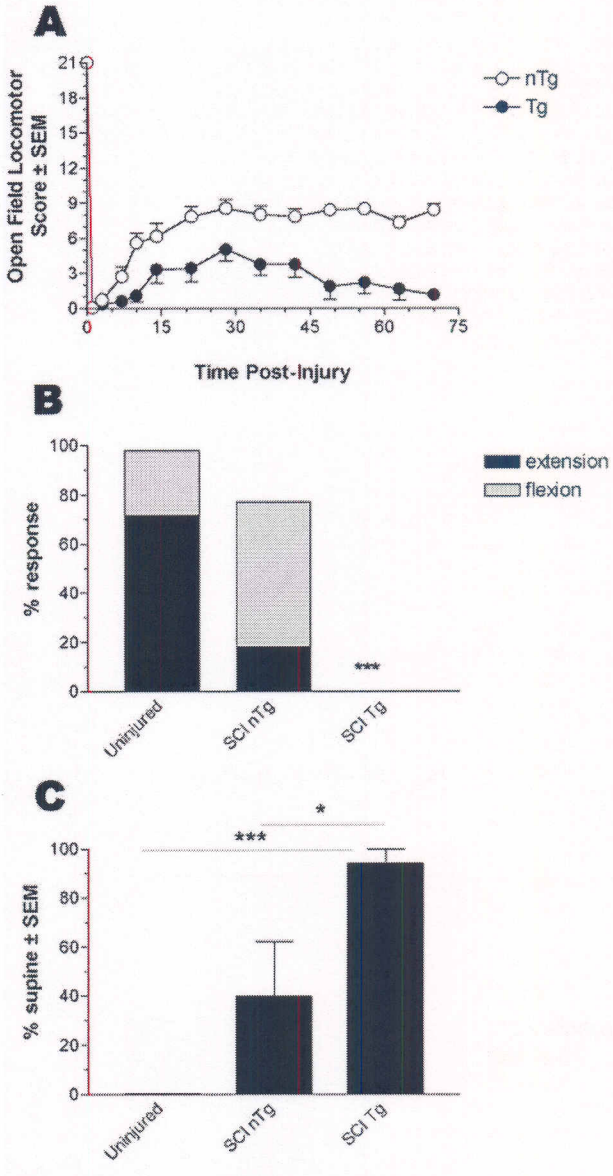

Figure 1. Neurological deficits are exacerbated in MBP TCR Tg mice after SCI. Motor function was consistently impaired in SCI–Tg mice (p < 0.001) compared with SCI–nTg controls (*A*). The integrity of spinal segmental circuitry (assessed by proprioceptive placing) and vestibulospinal innervation of the lumbar spinal cord (assessed by air righting) also was significantly impaired in SCI–Tg mice (*B* and *C*, respectively). SCI–Tg mice failed to exhibit the proprioceptive placing reflex (***p < 0.001 vs uninjured and SCI–nTg mice), whereas injured-nTg littermates exhibited the flexion phase of the reflex (*gray*). Uninjured littermates exhibited both the flexion and extension phases (*black*) of the reflex. Analysis of the air-righting reflex (*C*) revealed that uninjured mice rotate to prone and never land supine. SCI–nTg mice demonstrate hindquarter rotation and infrequent supine landing (not significant from uninjured control), illustrating the disruptive effect of SCI on the righting reflex. SCI–Tg mice land supine most of the time (>80%), exhibiting greater deficits than SCI–nTg (*p < 0.05) and uninjured (***p < 0.001) mice.

variable G and set to a value of 0 for SCI–nTg mice and to a value of 1 for SCI–Tg mice. Thus, the full model can be represented as follows:

$$Y_{id} = a_i - a_i \exp^{-(d_i-1)c_i} + e_{id} \quad (2)$$

$$a_i = a_0 + w_a G_i + u_{a_i} \quad (3)$$

$$c_i = c_0 + w_c G_i + u_{c_i} \quad (4)$$

In these equations, subscripts i and d represent individual animal and day, respectively. The first equation represents the basic model for change in BBB over time, and the second and third equations are linear equations representing asymptote and initial rate as a function of group, with residuals u_a and u_c, respectively. In fitting this model to the observed data, parameters of primary interest were a_0, c_0, w_a and w_c. It is straightforward to show that a_0 and c_0 will be estimates of the mean asymptote and initial rate for SCI–nTg mice (coded as $G_i = 0$). Coefficients w_a and w_c will represent effects of group on asymptote and initial rate and will be equal to the difference between SCI–nTg and SCI–Tg mice in mean asymptote and initial rate, respectively. The model was fit to the observed data using the NLMIXED procedure of SAS (1999). Using this model, the group difference in mean asymptotes was estimated to be −4.32, which was statistically significant ($t_{(16)} = -3.67$; $p < 0.001$). This result indicates that SCI–nTg mice reached a significantly higher level of recovery than did SCI–Tg mice. Results also showed a slightly more rapid initial rate of recovery for SCI–nTg mice, although this difference was not significant ($t_{(16)} = -1.31$; $p = 0.21$).

For proprioceptive placing, the number of trials of 10 in which flexion and extension, flexion only, or no hindlimb response was elicited was compared using MANOVA. For air righting, the percentage of four trials in which supine landing occurred for each mouse was recorded and analyzed with a one-way ANOVA followed by Bonferroni *post hoc* comparisons. Cytokine mRNA and ELISPOT data were evaluated using two-way ANOVA (group and time after injury as the two factors) followed by Tukey *post hoc* comparisons. Neuroanatomical outcome measures were evaluated using unpaired t tests to compare group differences. Results were considered statistically significant at $p < 0.05$.

RESULTS

Neurological dysfunction is exacerbated in spinal injured MBP TCR Tg mice

A contusion injury was induced in mice at the level of the midthoracic spinal cord, after which motor recovery was assessed over a period of 10 weeks using a standardized rating scale (Basso et al., 1995) (Fig. 1*A*). Gradual improvements in hindlimb function were observed in SCI–nTg littermates, culminating in consistent and complete flexion of hip, knee, and ankle joints. A majority of SCI–nTg mice ($n = 8$ of 9) regained the ability to weight-support and step. In contrast, functional recovery in SCI–Tg mice was consistently impaired as compared with the SCI–nTg group ($p < 0.001$) (Fig. 1*A*). Hindlimb flexion was limited or absent at all times examined and SCI–Tg mice never regained the ability to step or weight support. To evaluate the integrity of segmental spinal circuitry and descending brainstem innervation of the spinal cord, proprioceptive placing and air righting reflexes were tested in normal (uninjured), SCI–nTg, and SCI–Tg mice (Fig. 1*B,C*). Lumbar segmental reflexes, determined by proprioceptive placing, were absent in all SCI–Tg mice ($p < 0.001$) (Fig. 1*B*). Response frequency in SCI–nTg mice was not significantly different from uninjured control mice ($p = 0.55$). The air righting reflex, mediated by the vestibulospinal system (Pellis et al., 1991), was significantly impaired in all spinal injured animals ($p < 0.001$) with the greatest deficits present in SCI–Tg mice ($p = 0.03$) (Fig. 1*C*). Injured Tg mice had a nearly complete loss of hindquarter rotation and a concomitant increase in supine rather than prone landing (Fig. 1*C*).

Secondary injury is exacerbated, and axon sprouting is impaired in transgenic mice

Impaired functional recovery correlated ($p = 0.005$; $r^2 = 0.696$) with greater loss of myelinated axons at the impact site (mean sparing of spinal cord white matter decreased from ~100 μm^2 in SCI–nTg mice to <50 μm^2 in SCI–Tg mice; $p < 0.05$) and increased rostrocaudal lesion expansion in SCI–Tg mice (Fig. 2). In SCI–nTg mice, we observed typical patterns of demyelination. Specifically, demyelination rostral to the impact site (2 mm) was restricted to the dorsal funiculus, a region of the spinal cord containing axons undergoing Wallerian degeneration (Fig. 3*A,B*). Myelin and large-caliber axons of the lateral funiculi were intact (Fig. 3*E,F,I*). In contrast, we observed significant demyelination and axon loss throughout the white matter of SCI–Tg mice (Fig. 3*C,D,G,H,J*). Lymphocytes (CD3+) were localized to these regions of myelin–axon pathology only in SCI–Tg animals (Fig. 3*K*). This pathology is reminiscent of the T-cell-mediated autoimmune demyelination frequently observed in EAE.

A densely packed, fibronectin-rich connective tissue mass, a common pathological feature of the injured mouse spinal cord (Jakeman et al., 2000), was evident at the impact site in SCI–nTg mice (Fig. 4*A*). In contrast, small necrotic cavities and a loosely organized fibrous tissue matrix were observed in SCI–Tg mice (Fig. 4*B*). Differences in the matrix were associated with significantly less axon sprouting at the impact site in SCI–Tg mice ($p < 0.05$) (Fig. 4*C,D*). Thus, the integrity of the tissue at the impact site and the permissiveness of this substrate to support endogenous axon growth are dramatically decreased in SCI–Tg animals. Given that the force and related biomechanical variables (i.e., displacement distance, impulse, and momentum) associated with the injury were identical between groups, the destructive effects of the mechanical trauma (e.g., shear forces on axons and microvasculature) should be similar between groups. Taken together, sustained neurological impairment and the quantitatively larger tissue pathology observed in SCI–Tg mice imply "secondary injury" precipitated by injury-induced activation of MBP-reactive T-cells resulting in the onset of a pathological autoimmune response.

Cytokine production in peripheral lymphoid tissues after spinal cord injury

To evaluate whether SCI represents a sufficiently strong signal for peripheral lymphocyte activation, we analyzed splenic lymphocyte cytokine production at 3, 7, and 21 dpi, times previously shown to be associated with endogenous T-cell activation after SCI in rats (Popovich et al., 1996, 2001). As expected, lymphocyte cytokine production was minimal or absent in uninjured and SCI–nTg mice at all time points examined and is consistent with the low frequency of MBP-reactive T-lymphocytes in normal (nTg) mice and rats (Popovich et al., 2001) (Fig. 5). In contrast, markedly increased numbers of Th1 and Th2 cytokine-producing lymphocytes were found in SCI–Tg mice. Specifically, the frequencies of IL-2- and IFN-γ-producing cells (Th1) were elevated at all times in SCI–Tg mice compared with SCI–nTg mice ($p < 0.0001$ and $p = 0.0105$, respectively). At 7 dpi (IL-2), and at 7 and 21 dpi (IFN-γ), Th1-producing T-cells were elevated in SCI–Tg mice compared with uninjured Tg mice. There was a significant effect of group in frequency of IL-4- and IL-5-producing T-cells (Th2 cells; $p < 0.0001$ and $p < 0.01$, respectively), indicating that the number of cells producing Th2 cytokines was increased in SCI–Tg mice compared with SCI–nTg mice. T-cell production of the immune-regulatory cytokine, TGF-β, was below the levels of detection by ELISPOT (data not shown). Lymphocyte production of IL-10, a cytokine with neuroprotective potential after SCI (Bethea et al., 1999), was significantly decreased in SCI–Tg mice

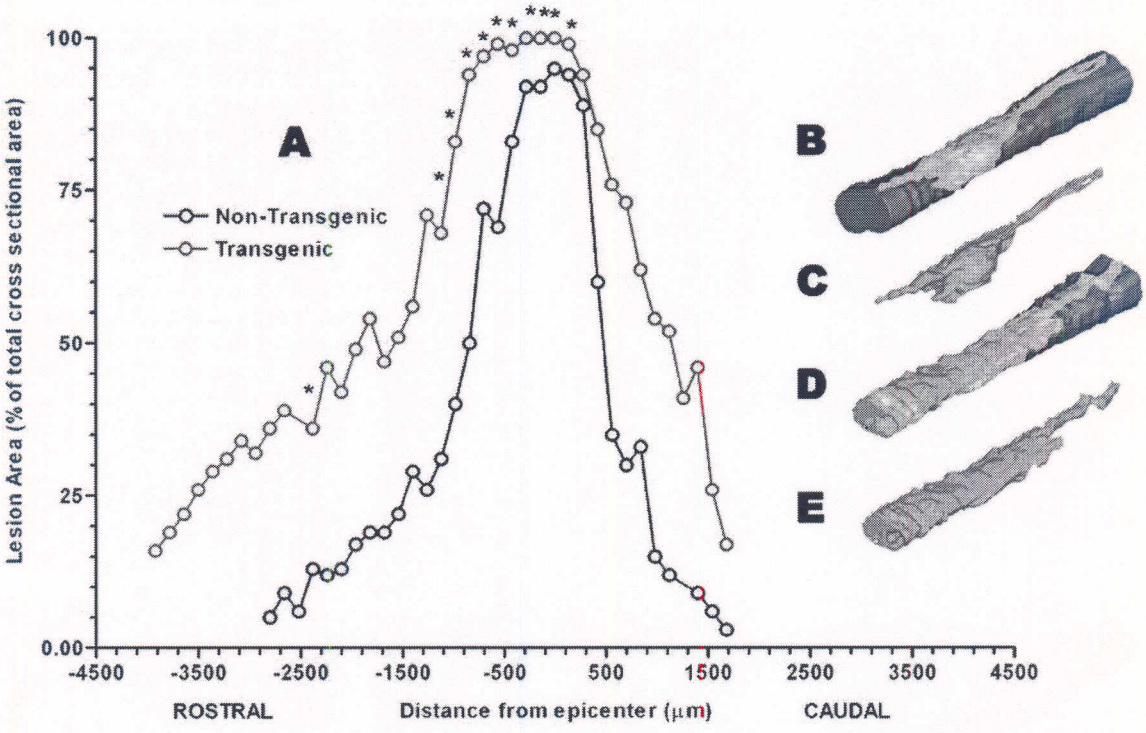

Figure 2. Proximal–distal lesion expansion is increased in MBP TCR Tg mice after SCI. The area occupied by lesion, defined as the absence of normal spinal cord architecture and the presence of fibrous tissue or demyelination, is greater throughout the rostrocaudal extent of SCI–Tg spinal cord compared with SCI–nTg controls (*$p < 0.05$ at indicated levels). Data are expressed as a percentage of the total cross-sectional area at a given level. Total lesion area (assessed by area under the curve) was significantly different between the two groups ($p < 0.05$). Three-dimensional reconstructions of injured spinal cords from representative SCI–nTg (*B, C*) and SCI–Tg (*D, E*) mice illustrate the magnitude of differences in the lesion pathology. *Green shading* depicts lesioned tissue in SCI–nTg (*B, C*) and SCI–Tg mice (*D, E*).

($p < 0.05$; 377.7 ± 90 pg/ml in uninjured vs 155 ± 44 pg/ml in SCI–Tg mice; $n = 6$ and 4, respectively).

Impaired functional recovery and exacerbated neuropathology in MBP TCR Tg mice are associated with increased intraspinal proinflammatory cytokine mRNA

Increased trafficking of autoreactive T-lymphocytes from the periphery to the spinal cord in SCI–Tg mice could account for the exacerbated neuropathology and neurological dysfunction described above. Thus, mRNA profiles for intraspinal cytokines were evaluated at 7 and 21 dpi, i.e., times that corresponded with peripheral T-cell activation (see above) and with the onset and plateau of behavioral differences between the two groups of animals (Fig. 1*A*).

IL-2 is produced by activated T-cells after antigen stimulation. Thus, we measured IL-2 mRNA within the injured and uninjured spinal cord of Tg and nTg mice as an index of intraspinal T-cell activation. Significant IL-2 mRNA was detectable in SCI–Tg mice only ($p < 0.05$) (Fig. 6).

To determine whether the neurological impairments observed in SCI–Tg mice were associated with differential intraspinal production of proinflammatory (Th1) cytokines, we evaluated IL-1β, tumor necrosis factor (TNF) α, IL-6, IL-12, and IFN-γ mRNA in nTg and Tg mice (Fig. 6). Similar to our previous characterization of cytokine changes in injured rat spinal cord (Streit et al., 1998), we noted an increase in cytokine mRNA in the injured spinal cord of both nTg and Tg mice. However, the magnitude of the cytokine response was always greater in SCI–Tg mice. Specifically, IFN-γ, IL-1β, and TNFα mRNA were increased in Tg compared with nTg mice at 7 and 21 dpi ($p < 0.01$). IL-12 mRNA was significantly increased at 21 dpi only in Tg mice ($p < 0.01$). Increased IL-6 mRNA was also detected in SCI–Tg mice but values did not reach statistical significance ($p = 0.0699$).

Because the neuroprotective potential of MBP-reactive T-cells has been associated with their ability to produce neurotrophins and immune-regulatory (Th2) cytokines (Moalem et al., 2000a; Yoles et al., 2001), we assessed whether neurotrophin (NT)-3, BDNF, IL-4, IL-5, and IL-10 mRNA were differentially expressed in the injured spinal cord of Tg and nTg mice (Fig. 7). Overall, patterns of neurotrophin and Th2 cytokine mRNA expression were similar between groups. There was no significant effect of group for either BDNF or NT-3. BDNF and NT-3 mRNAs were significantly reduced at 7 dpi in both Tg and nTg mice ($p < 0.05$). Whereas BDNF mRNA returned to uninjured control values by 21 dpi, NT-3 remained below baseline values ($p = 0.05$). There was no effect of group or time for IL-4 mRNA expression. However, significant differences in IL-5 and IL-10 mRNA expression were noted between groups. Although IL-5 mRNA was significantly increased at 21 dpi in both groups of mice, values were increased to a greater extent in Tg mice ($p < 0.001$ vs nTg mice). Similarly, IL-10 mRNA was increased in both groups of mice at 7 and 21 dpi, with Tg mice exhibiting a greater increase at both time points ($p < 0.05$ and $p < 0.0001$ vs nTg mice, respectively).

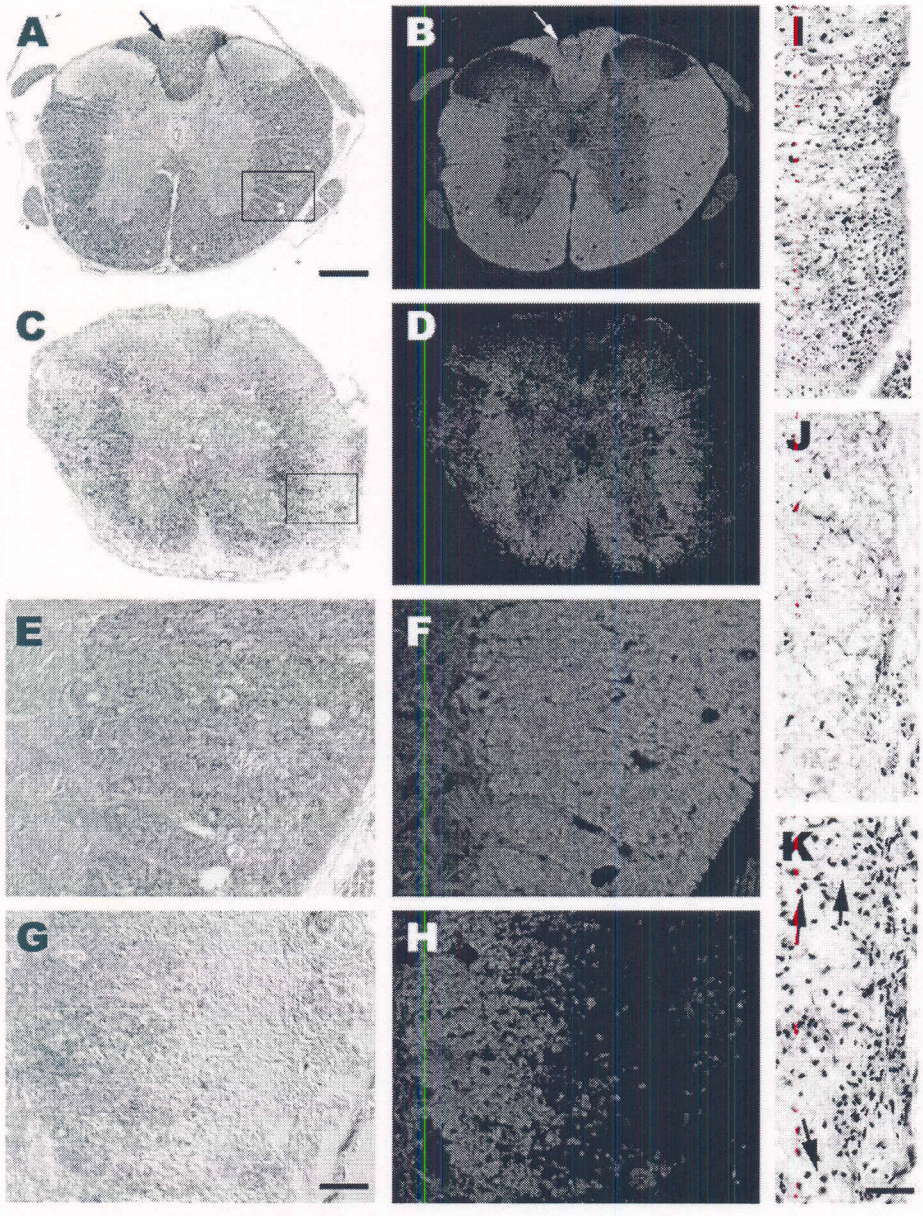

Figure 3. Lymphocytes localize to regions of demyelination and axon loss after SCI in MBP TCR Tg mice. In SCI–nTg mice, demyelination was restricted to the dorsal funiculus (*A, B, arrows;* 2 mm rostral to impact site). Myelin (LFB; *E, F*) and large-caliber axons (anti-neurofilament; *I*) in lateral funiculi were intact. In contrast, significant demyelination (*C, D*) and axon loss (*J*) were observed throughout the white matter of SCI–Tg mice. Lymphocytes (anti-CD3) were localized to regions of myelin–axon pathology only in SCI–Tg animals (*K*). *Boxed regions* in *A* and *C* shown in high power in *E–H*. The contrast of typical light-microscopic images of LFB histochemistry (*A, C, E, G*) were enhanced by viewing the same sections with dark-field (*B, D, F, H*) microscopy together with a rhodamine filter set. Scale bars: *A–D,* 230 μm; *E–H,* 56 μm; *I–K,* 40 μm.

DISCUSSION

Despite decades of research implicating CNS autoreactive lymphocytes as effectors of neuropathology and disease [e.g., multiple sclerosis (MS) and EAE], recent studies have suggested these cells can minimize the delayed neuronal and glial cell death that accompanies CNS trauma. Specifically, neuroprotection and functional recovery were observed after spinal cord contusion or optic nerve crush when the cellular immune response was boosted via a CNS-specific vaccine or if the animal's immune system was supplemented with exogenous myelin-reactive T-cells (Moalem et al., 1999a, 2000b; Hauben et al., 2000a, 2001). Additional data suggests that myelin-reactive T-cells are present normally in animals but that the neuroprotective autoimmune response elicited by trauma is insufficient or is downregulated by the CNS microenvironment (Cohen and Schwartz, 1999; Schwartz et al., 1999b; Yoles et al., 2001). Consequently, we reasoned that tissue preservation and recovery from SCI would be markedly improved in an animal model in which augmentation of myelin-reactive T-cells is unnecessary, i.e., a Tg mouse with a large endogenous repertoire of CNS autoreactive T-cells (>95% of all CD4+ T-cells). Instead, we observed impaired recovery of locomotor and reflex function in SCI–Tg mice compared with SCI–nTg littermates. Functional impairment correlated with exacerbated secondary degeneration and demyelination as well as increased intraspinal expression of proinflammatory cytokine mRNA in SCI–Tg mice. The present results illustrate the complexities of interpreting and implementing therapies designed to activate CNS autoreactive T-cells. Indeed, a number of poorly characterized biological variables that influence T-cell activation and the development of immune responses within the injured spinal cord need to be defined before CNS vaccines can be safely implemented.

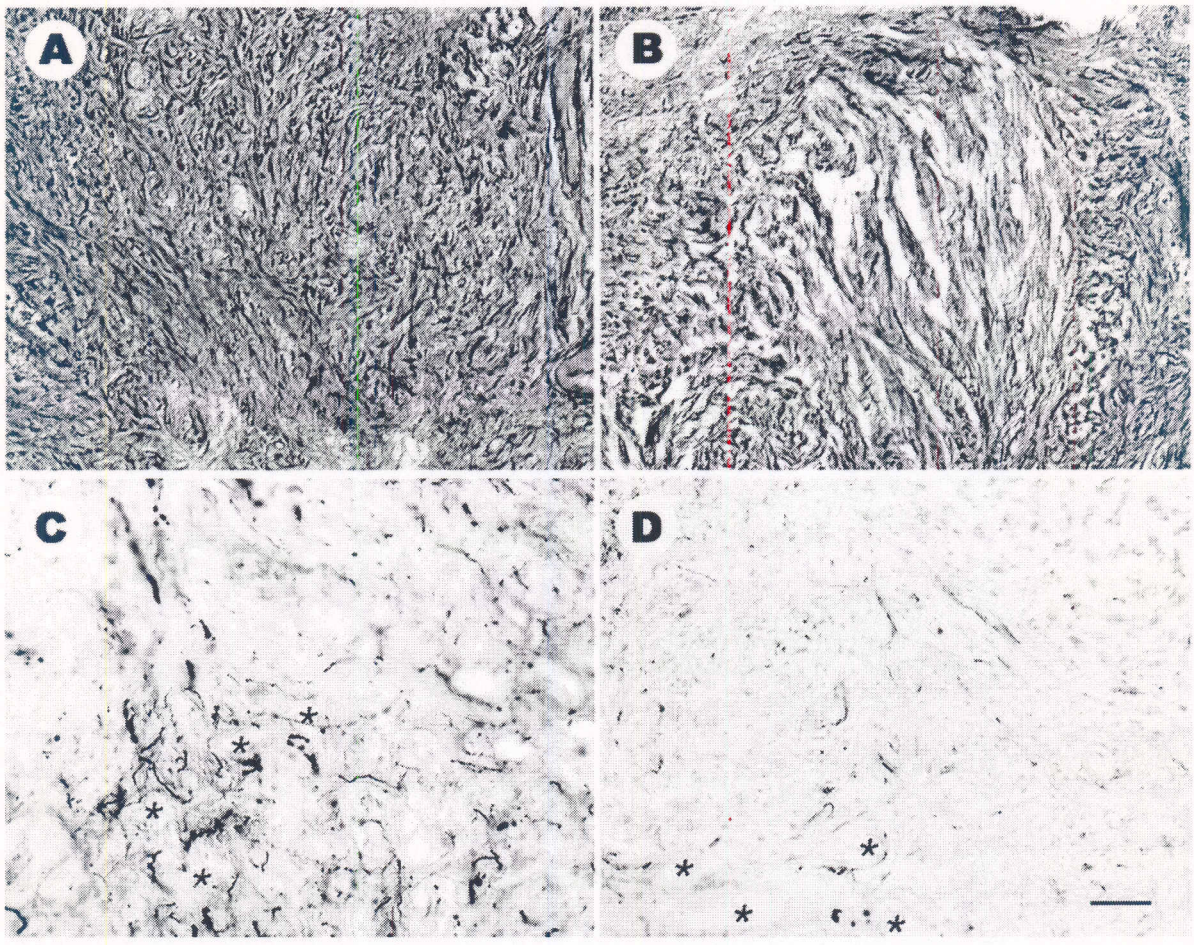

Figure 4. Reduced axon growth in the center of the contusion lesion is associated with altered matrix deposition after SCI in MBP TCR Tg mice. The dense fibronectin-rich connective tissue matrix, characteristic of mouse SCI, appears normal in SCI–nTg mice (*A*) but loosely organized in SCI–Tg mice (*B*). Axon growth and sprouting (anti-neurofilament) in these same regions is pronounced in SCI–nTg animals (*C*) but is significantly reduced ($p < 0.05$; $n = 4$ per group) in SCI–Tg mice (*D*). *Asterisks* indicate blood vessel profiles. Scale bar, 100 μm.

For CNS-reactive T-cells to support neuronal and glial survival after SCI, these cells need to be present during a time that precedes or overlaps the onset of secondary degeneration. Moreover, these cells would have to directly or indirectly provide neurons and glia with trophic molecules to support their survival. Although infiltration of endogenous T-cells precedes secondary demyelination after SCI (Popovich et al., 1997), the antigen-specificity and functional potential of these T-cells is either poorly defined or is unknown. Furthermore, even if a subset of these T-cells react with CNS proteins, CNS-reactive T-cells are likely to be suppressed or killed in the target organ (i.e., brain or spinal cord) (Smith et al., 1996), or their frequency will be too low to promote a functionally significant response (Moalem et al., 1999b; Yoles et al., 2001). Even in models of T-cell-mediated autoimmune disease (i.e., EAE) in which animals are immunized with myelin antigens, most T-cells that infiltrate the CNS do not react with CNS proteins (Cross et al., 1990; Steinman, 1996). Thus, to enhance the reactivation of neuroantigen-specific T-cells within the target organ, a necessary prerequisite for the induction of large quantities of T-cell-derived neurotrophic cytokines or growth factors (e.g., BDNF, NT-3, NGF) (Kerschensteiner et al., 1999; Hammarberg et al., 2000; Moalem et al., 2000a), vaccination would appear to be a logical approach. However, the identity and functional potential of the "neuroprotective" cell type have not been determined, and it is essential that future studies examine the possibility that neuroprotection is mediated by other leukocytes recruited to the CNS with myelin-reactive T-cells.

In the present study, most (>95%) of the CD4+ T-lymphocytes in the Tg mice react with MBP (Olivares-Villagomez et al., 1998). These cells, after reactivation with MBP, produce prodigious amounts of neurotrophins *in vitro* (Moalem et al., 2000a). However, in our model, the lack of induction of NT-3 or BDNF mRNA in Tg mice suggests that MBP-reactive T-cells are not a primary source of neurotrophins after SCI. Previous studies by Hammarberg et al. (2000) suggest that the key effectors of neurotrophin release *in vivo* are non-CNS-reactive T-cells (i.e., bystander or regulatory T-cells) or NK cells that are recruited to the injury site with MBP-reactive T-cells. Because the Tg mice used in the current studies have few regulatory T-cells, any neurotrophic contributions of these cells would be masked in our model. Thus, in the absence of sufficient regulation, overwhelming the CNS with too many proinflammatory, autoreactive T-cells may do more harm than good. In support of this, the neuroprotection afforded by immunization with MBP is abolished if the

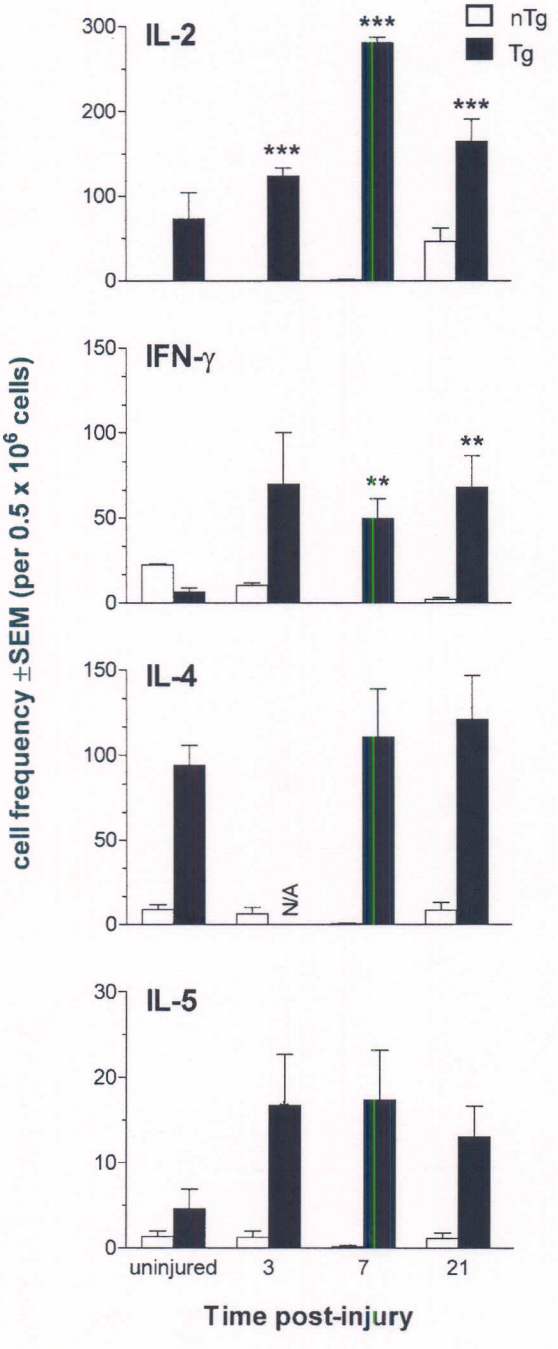

Figure 5. MBP-reactive T-cells are activated in the periphery by SCI and produce Th1 and Th2 cytokines. There was a significant effect of group for all cytokines assessed, indicating that the frequency of Th1- and Th2-producing T-cells is increased in SCI–Tg mice compared with SCI–nTg mice ($p < 0.01$). Increased frequency of IL-2-producing lymphocytes in the spleen at 3, 7, and 21 dpi indicates peripheral activation of MBP-reactive T-cells in SCI–Tg mice (***$p < 0.001$ vs nTg). Similarly, the frequency of cells producing IFN-γ in SCI–Tg mice was increased at 7 and 21 dpi compared with uninjured Tg mice (**$p < 0.01$ and $p = 0.01$, respectively). There was no significant effect of time for either IL-4 or IL-5, indicating that, despite higher frequencies of these cytokine-producing cells in the SCI–Tg mice compared with SCI–nTg mice, the frequency of Th2-producing cells was not increased after SCI. Although not statistically significant, there was a trend toward an increase in IL-5-producing T-cells at all time points assessed.

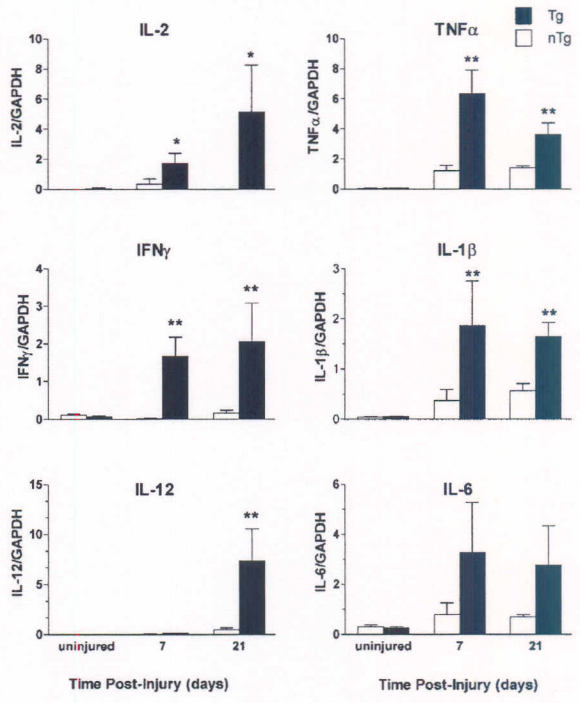

Figure 6. Proinflammatory (Th1) cytokine mRNA expression is enhanced in the injured spinal cord of MBP TCR Tg mice. IL-2 mRNA is increased (*$p < 0.05$ vs nTg) in SCI–Tg but not SCI–nTg mice, indicating that T-cells infiltrating the injured spinal cord are activated. Minimal induction of proinflammatory cytokine mRNA is present in uninjured spinal cord of Tg and nTg mice. However, IFN-γ, IL-1β, and TNFα mRNA are significantly increased at 7 and 21 dpi only in SCI–Tg mice (**$p < 0.01$ vs nTg). Note that IL-12 mRNA is markedly increased only in SCI–Tg mice at 21 dpi (**$p < 0.01$ vs nTg).

bacterial content of the immunizing adjuvant is too high (Hauben et al., 2001). High bacterial concentrations preferentially activate large numbers of Th1 lymphocytes producing IL-2, IFN-γ, TNFα, and IL-12. These cytokines have devastating effects in the CNS (Martin et al., 1992). Similarly, although neuroprotective at low concentrations, MBP-activated lymphocytes lose their neuroprotective effects when injected into SCI rats in increasingly larger doses (Yoles et al., 2001).

The environment of the injured CNS affects the phenotype of autoreactive T-cells

Previously, we demonstrated that MBP-activated lymphocytes isolated from spinal injured rats were capable of producing a mild, transient paralytic disease when injected into naive (uninjured) rats (Popovich et al., 1996). However, using a similar approach, MBP-reactive T-cells taken from SCI animals were found to be neuroprotective when injected into normal rats that were subsequently injured (Yoles et al., 2001). Together, these studies suggest that SCI activates myelin-reactive T-cells but does not guarantee their neuroprotective potential will be realized; this appears to depend on other cells or the CNS microenvironment. The microenvironment that is created within peripheral lymphoid tissues or the spinal cord as a result of immunization will determine the functional potential of antigen-specific T-cells, i.e., whether they become pathogenic Th1 or immunoregulatory Th2 lymphocytes (Irani et al., 1997; O'Garra and Arai, 2000; Rengarajan et al., 2000). Our current findings indicate that the

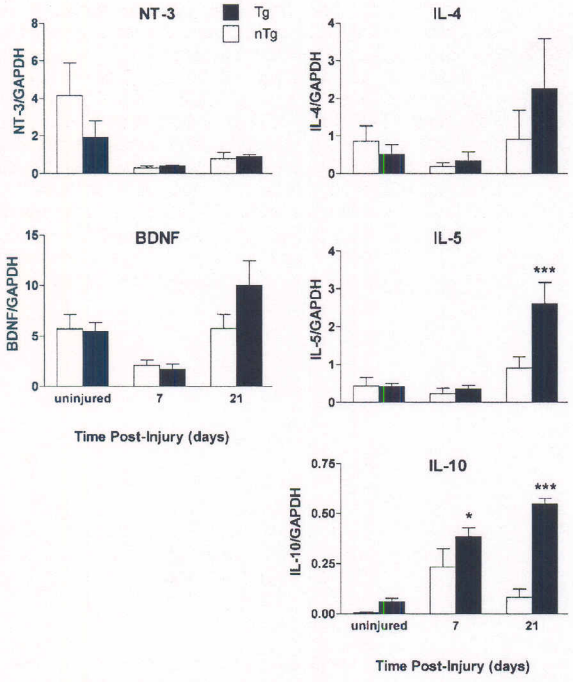

Figure 7. Neurotrophin and immunoregulatory (Th2) cytokine mRNA profiles in the spinal cord of Tg and nTg mice after contusion injury. BDNF and NT-3 mRNA decreased at 7 dpi with no differences observed between SCI–Tg and SCI–nTg mice. Although BDNF mRNA returned to baseline by 21 dpi, NT-3 remained below uninjured levels at 21 dpi ($p = 0.05$). Expression of IL-4 mRNA did not change over time or vary between groups. However, there was a significant increase in IL-10 mRNA expression in SCI–Tg mice compared with SCI–nTg mice at 7 dpi ($*p < 0.05$) and in IL-5 and IL-10 mRNA expression at 21 dpi ($***p < 0.001$ vs nTg).

environment at the injury site influences MBP-reactive T-cell function. For example, although MBP-reactive T-cells in the periphery of SCI–Tg mice appear multipotent (i.e., both Th1 and Th2 lymphocytes are activated), proinflammatory Th1 cytokine mRNAs are increased to a greater extent (e.g., 5- and 240-fold increase for IL-12 or 99- and 56-fold increase for TNFα at 7 and 21 d, respectively) than immunosuppressive Th2 cytokine mRNA (e.g., sixfold and ninefold increase of IL-10 at 7 and 21 dpi). If more regulatory T-cells were recruited to the injury site, perhaps the increased expression of neurotrophins would mollify the destructive potential of proinflammatory cytokines as was shown previously (Hammarberg et al., 2000). Unfortunately, the decrease of all tyrosine receptor kinase receptors at or nearby the injury site during the first week after injury would preclude the effectiveness of any neurotrophin molecules produced by infiltrating T-cells (Liebl et al., 2001). Consequently, if neurotrophins underlie beneficial autoimmunity, perhaps it is a result of their ability to directly interfere with the destructive effects of proinflammatory molecules. If so, neurotrophins, in addition to their roles in promoting axonal sprouting or regeneration and neuronal survival, may serve as feedback regulators of the immune response (Heese et al., 1998). Taken together, the decline of intraspinal NT-3 and BDNF mRNA and the large induction of IFN-γ, TNFα, IL1-β, IL-6, and IL-12 mRNA signifies an environment in which inflammatory-mediated injury predominates.

Proinflammatory cytokines and autoimmune neuropathology

The mechanisms that underlie the marked behavioral and neuropathological differences that we have described between SCI–Tg and SCI–nTg mice could be explained by the overwhelming increase in the Th1:Th2 cytokine ratios. Indeed, each of the Th1 cytokines that we evaluated are associated with demyelination, axonal injury, or neuronal cell death (Merrill and Benveniste, 1996; Allan et al., 2001). That some of these molecules (e.g., IL-1, IL-6, TNFα) have been associated with processes of neural repair, remyelination, and/or revascularization (Schwartz et al., 1991; Mason et al., 2001) and are expressed in both Tg and nTg mice, albeit to different levels, emphasizes how critical it is that we learn how to control their production in the context of an ongoing T-cell response. Future studies must consider the microenvironment in which the T-cells are activated and how intercellular reactions at the injury site influence T-cell secretory potential.

The selective induction of IL-2, IFN-γ, and IL-12 in SCI–Tg mice is likely involved in mediating the functional disturbances and pathology observed in SCI–Tg mice. Within the CNS, release of IL-2 and IFN-γ by activated Th1 cells is associated with microglial–macrophage production of IL-12 (Krakowski and Owens, 1997). Moreover, IL-12 polarizes the T-cell secretory profile such that proinflammatory cytokines predominate. Targeted upregulation of IL-12 in the normal CNS produces a prominent Th1 lymphocyte reaction accompanied by axonal injury and demyelination (Lassmann et al., 2001). In MS, disease progression and the appearance of new demyelinating plaques is associated with increased IL-12 (Balashov et al., 1997). The profile of cytokine expression described in SCI–Tg mice at 7 and 21 dpi is similar to what is observed in EAE and MS (Ahmed et al., 2001) and demonstrates that CNS trauma can precipitate the onset of autoimmune pathology (Poser, 1994).

It is interesting that Yoles et al. (2001) reported increased survival of retinal ganglion cells after optic nerve crush using the same MBP TCR Tg mice described in the present study. The disparity between our results and theirs could be explained by unique immune networks in the brain and spinal cord. For example, similar traumatic injuries to the cerebral cortex and spinal cord result in a greater magnitude and duration of blood–brain barrier injury and leukocyte recruitment in the spinal cord (Schnell et al., 1999a). This may be explained by the divergent patterns of cytokine–chemokine signaling in these CNS compartments (Bell et al., 1996; Schnell et al., 1999b). Thus, greater trafficking of MBP-reactive T-cells and other leukocytes to the injured spinal cord compared with injured optic nerve could result in a ratio of pathogenic to regulatory T-cells that is incompatible with neuroprotection. Further studies are needed to establish the extent to which mechanisms of immune regulation are distinct between brain and spinal cord. If they are numerous and unique, the development of immune-based therapies for brain- (e.g., Alzheimer's disease) or spinal cord-specific (e.g., spinal trauma) disease may require dedicated brain–spinal cord models before successful clinical application becomes feasible.

CNS trauma and autoimmune disease

Experimental and clinical nerve trauma (either accidental or introduced by surgical intervention) can cause the expansion of myelin-reactive lymphocytes (Olsson et al., 1992, 1993; Kil et al., 1999). The present studies demonstrate, for the first time, the potential for a causal relationship between CNS trauma and the

onset of autoimmune pathology in genetically susceptible animals. Because of the marked divergence in motor recovery early after injury that was sustained for the duration of these studies, it is unlikely that spontaneous autoimmune disease accounts for the distinct behavioral differences observed. In fact, a small percentage of CD4+ regulatory T-cells make this strain of Tg mouse resistant to spontaneous autoimmune disease (Lafaille et al., 1994; Van de Keere and Tonegawa, 1998). However, these mice, like a subset of humans, are predisposed toward CNS autoimmune disease if the MBP-reactive T-cells are appropriately activated. As described in this study, SCI is sufficient to deliver this activation signal.

Still, T-cell-mediated neuroprotection after CNS trauma may be inversely related to an individual's susceptibility to autoimmune disease. Indeed, Kipnis et al. (2001) have shown that resistance to CNS autoimmune disease and protective T-cell-dependent immunity are closely related. Thus, safe and functionally significant vaccine approaches will require previous knowledge of an individual's predisposition to developing autoimmune disease. Given that genetic and environmental factors contribute to autoimmune disease, various obstacles will need to be overcome before clinical trials should be attempted.

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