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Role of passive T-cell death in chronic experimental autoimmune encephalomyelitis

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The mechanisms of chronic disease and recovery from relapses in experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis, are unknown. Deletion of myelin-specific lymphocytes by apoptosis may play a role in termination of the inflammatory response. One pathway of apoptosis is the passive cell death or “cell death by neglect” pathway, which is under the control of the Bcl family of genes. To investigate the role of passive cell death pathway in EAE, we used mice with transgenic expression of the long form of the bcl-x gene (Bcl-xL) targeted to the T-cell lineage. We found that mice transgenic for Bcl-xL have an earlier onset and a more chronic form of EAE induced by myelin oligodendrocyte glycoprotein (MOG) peptide 35–55 compared with wild-type littermate mice. This was not due to an expanded autoreactive cell repertoire. Primed peripheral lymphocytes from Bcl-xL transgenic mice showed increased proliferation and cytokine production to MOG peptide in vitro compared with lymphocytes from wild-type animals. Immunohistologic studies demonstrated increased cellular infiltrates, immunoglobulin precipitation, and demyelination in the Bcl-xL transgenic central nervous system (CNS) compared with controls. There was also a decreased number of apoptotic cells in the CNS of Bcl-xL transgenic mice when compared with littermates at all time points tested. This is the first report of an autoimmune disease model in Bcl-xL transgenic mice. Our data indicate that the passive cell death pathway is important in the pathogenesis of chronic EAE. These findings have implications for understanding the pathogenesis of multiple sclerosis and other autoimmune diseases.

is augmented and prolonged by CD28 costimulation (4, 14–16). In Bcl-xL transgenic mice, Bcl-xL expression is detectable in unstimulated T cells and is constitutively expressed after activation of T cells with or without CD28 costimulation (3). Thymocytes from Bcl-xL transgenic mice are resistant to apoptosis induced by glucocorticoids, γ-irradiation, calcium ionophore, and CD3 cross-linking, but they are not protected from clonal deletion by self-antigens (3). We have previously found that transgenic expression of Bcl-xL does not obviate the requirement for CD28 signaling in T-cell proliferation, and although Bcl-xL expression protects T cells from apoptosis resulting from growth factor insufficiency, they were still sensitive to AICD (17).

In this report, we found that Bcl-xL transgenic mice immunized with myelin oligodendrocyte glycoprotein peptide 35–55 (MOG p35–55) developed more severe EAE than nontransgenic littermates. This was associated with increased antigen-specific proliferation, cytokine production by primed T cells, and more severe pathology in the CNS. More importantly, the Bcl-xL transgenic mice had fewer apoptotic cells in the CNS compared with littermates at all time points tested. These data underscore the importance of T-cell apoptosis in terminating the inflammatory response in EAE and suggest that failure to terminate T-cell responses in the CNS may be a mechanism for the development of chronic disease.

Methods

Mice. Bcl-xL transgenic mice originally provided by G. Nunez (University of Michigan, Ann Arbor, Michigan, USA) were back-crossed onto a B6 background for 9 generations. The animals were bred in our facility and were screened for the presence of the transgene by PCR. The primers were forward: GCGGGCATTCAGTGACCTGA, and reverse: TAAGTGGCCATCCAAGCTGC. Sex and age-matched wild-type littermates were used as controls (17).

EAE induction with MOG peptide. MOG p35–55 (M-E-V-G-W-Y-R-S-P-F-S-R-O-V-H-L-Y-R-N-G-K) corresponding to mouse sequence was synthesized by Quality Controlled Biochemicals Inc. (Hopkinton, Massachusetts, USA) and purified by HPLC. Peptide purity was greater than 99% after HPLC. Experiments were performed with 3–8 animals in each group. Bcl-xL transgenic and wild-type littermates were immunized subcutaneously in the flanks with 200 μg of MOG peptide in 0.1 mL PBS and 0.1 mL CFA containing 0.4 mg Mycobacterium tuberculosis (H37Ra; Difco Laboratories, Detroit, Michigan, USA) and intraperitoneally injected with 200 ng Pertussis toxin (List Biological Laboratories Inc., Campbell, California, USA) on the day of immunization and 2 days later.

EAE was scored as previously described (18, 19): grade 1, limp tail or isolated weakness of gait without limp tail; grade 2, partial hind limb paralysis; grade 3, total hind limb or partial hind and front limb paralysis; grade 4, total hind limb and partial front limb paralysis; grade 5, moribund or dead animal. In C57/B6 mice, MOG-induced disease develops around day 15 (Table 1), and the mice undergo an acute phase lasting 10–12 days. Around 50% of the mice develop a persistent chronic disease at the end of the acute phase, which is characterized by a disease grade of 1 or more (20).

MBP and PLP immunization. Bcl-xL transgenic mice and wild-type littermates were immunized subcutaneously in the flanks with 100 μg of mouse myelin basic protein (MBP), prepared as previously described (21), emulsified in an equal amount of CFA containing 2 mg/mL M. tuberculosis (Difco Laboratories). The animals received a 200-ng dose of pertussis toxin intraperitoneally (List Biological Laboratories Inc.) 24 hours after immunization. Mice were immunized with proteolipid protein (PLP) (provided by V. Kuchroo, Brigham and Women’s Hospital, Boston, Massachusetts, USA) (100 μg per mouse emulsified in an equal amount of CFA containing 4 mg/mL M. tuberculosis). The mice received 100 ng of pertussis toxin intravenously on days 0 and 2 after immunization. Scoring of clinical disease was performed daily as above.

Staining for T-cell receptor variable β (TCR Vβ) chains. Splenocytes from naïve or MOG-immunized mice (on day 12 after immunization) were separated and resuspended...
in PBS to a concentration of 10⁷ cells/mL. Next, 100-μL aliquots were plated in a 96-well plate. The cells were treated with Fc Block (PharMingen, San Diego, California, USA) at a concentration of 1 μg/10⁶ cells for 5 minutes on ice. The cells were then stained with various Vβ FITC- or PE-conjugated antibodies (PharMingen): Vβ2 rat anti-mouse IgG2a PE, Vβ3 hamster anti-mouse IgG PE, Vβ4 rat anti-mouse IgG2b PE, Vβ5.1, 5.2 mouse anti-mouse IgG1 PE, Vβ6 rat anti-mouse IgG2b PE, Vβ7 rat anti-mouse IgG2b PE, Vβ8.1, 8.2 mouse anti-mouse IgG2a FITC, Vβ8.3 hamster anti-mouse IgG PE, Vβ9 mouse anti-mouse IgG1 FITC, Vβ10 rat anti-mouse IgG2a PE, Vβ11 rat anti-mouse IgG2b PE, Vβ12 mouse anti-mouse IgG1 FITC, Vβ13 mouse anti-mouse IgG1 PE, Vβ14 rat anti-mouse IgM FITC, Vβ17 mouse anti-mouse IgG2a, and TCR β chain constant region hamster anti-mouse FITC and PE. Appropriate isotype controls were used.

The cells were incubated on ice for 30 minutes, washed with 1% FBS in PBS twice, and then resuspended for analysis on a FACSscan equipped with CellQuest software (both from Becton Dickinson Immunocytometry Systems, San Jose, California, USA). Cells expressing specified Vβ type were plotted against the TCR constant region on dot plot, and percentages were calculated.

Cell culture. For in vitro cell culture experiments, mice were subcutaneously immunized in 1 hind footpad and in both flanks with an emulsion of 100 μL CFA and 100 μL PBS containing 100 μg MOG p35–55. The mice were sacrificed 10 days after immunization.

A single cell suspension was prepared from the inguinal and the draining popliteal lymph nodes or spleens. For proliferation and cytokine measurement, the cells were cultured in 96-well plates (Costar, Corning, New York, USA). Media used for proliferation and cytokine assays consisted of serum-free Ex-Vivo 20 medium (BioWhittaker Inc., Walkersville, Maryland, USA) containing 75 mM/mL L-glutamine, 100 U/mL penicillin and streptomycin, 1 mL/100 mL of a x100 concentrated non-essential amino acid solution, 0.1 mM HEPES/mL, 1 mM/mL sodium pyruvate (all from BioWhittaker Inc.), and 0.05 mM/mL 2-mercaptoethanol (Sigma Chemical Co.). Cells were incubated at 37°C in humidified air containing 7% CO₂.

Proliferation assay. The cells were cultured at 2 × 10⁶
B220+ B cells (B220), and immunohistochemically stained for activated macrophages (Mac-1), were collected when mice reached grade 2. The sections were after immunization with 200 μg of MOG p35–55. The spinal cords were then harvested with a Wallac 1205 Beta-plate Scintillation Counter (Wallac Oy, Turku, Finland) on filter mats and were then dried and counted.

**Cytokine ELISA.** The cells were cultured at 2 × 10^6 cells/mL in 200 μL media at various antigen concentrations. Supernatants for IL-10 and IFN-γ ELISA were collected after 48 hours of culture. Quantitative ELISAs for IL-10 and IFN-γ were performed using paired antibodies and recombinant cytokines from PharMingen according to the manufacturer’s recommendations.

**Staining for apoptosis of lymphocytes.** Cells were cultured in DMEM complete at a concentration of 2 × 10^6 cells/mL with MOG p35–55 at a concentration of 10 μg/mL in 6-well plates. The cells were harvested for TdT-mediated dUTP nick-end labeling (TUNEL) staining at 0, 24, 48, and 72 hours. The cells were washed and resuspended in PBS to a concentration of 10^7/mL, and then incubated on ice with rat anti-mouse CD4 PE-conjugated antibody (Caltag Laboratories Inc., Burlingame, California, USA) for 20 minutes. They were then washed with PBS and fixed in 4% paraformaldehyde for 30 minutes at room temperature. The cells were washed and permeabilized with 100 μL Triton X-100 in 0.1% saponin. 7-AAD stain was added to the plates at a concentration of 5 μg/mL to measure apoptosis in vitro. Staining of apoptotic cells with 7-AAD was done as described by Schmid et al. (22). Briefly, cells were prestained for surface antigen expression as above, were incubated with 20 μg/mL of 7-AAD in PBS-Az for 20 minutes at 4°C, and then were protected from light. Cells were then analyzed on FACSsort equipped with CellQuest software (both from Becton Dickinson Immunocytometry Systems).

We also used 7-amino-actinomycin D (7-AAD) staining (Calbiochem-Novabiochem Corp., La Jolla, California, USA) to measure apoptosis in vitro. Staining of apoptotic cells with 7-AAD was done as described by Schmid et al. (22). Briefly, cells were prestained for surface antigen expression as above, were incubated with 20 μg/mL of 7-AAD in PBS-Az for 20 minutes at 4°C, and then were protected from light. Cells were then analyzed on FACSort (Becton Dickinson Immunocytometry Systems) without further washing. Staining with 7-AAD differentiates cells with 7-AAD^dim^ that are in the early stages of apoptosis and 7-AAD^bright^ that are in the later stages of apoptosis (22).

**ELISPOT assay.** Cells (4 × 10^6/well) were incubated with antigen (as for the proliferation assays) in U-bottom plates for 24 hours. The cells were then counted, resuspended, and serially diluted from a concentration of 10^6 cells/mL down to 8 × 10^3 cells/mL, and then were added to nitrocellulose plates (Millipore, Bedford, Massachusetts, USA). The plates were coated with 50 μL primary IFN-γ-Ab (clone R4-6A2; Endogen Inc., Woburn, Massachusetts, USA) at a concentration of 5 μg/mL. Next, 100 μL of the appropriate concentrations of antigen or mitogen was added to the nitrocellulose ELISPOT plates. The cells were then incubated for an additional 18 hours at 37°C. After washing three times, 50 μL/well of biotinylated IFN-γ-secondary Ab (clone XMG1.2; Endogen Inc.) was added to the plates at a concentration of 2 μg/mL diluted in 1% BSA/PBS for 5 hours at room temperature. After washing, 50 μL of alkaline phosphatase (E-2636; Sigma Chemical Co.) was added to the plates at a dilution of 1:1,000 in 0.05% Tween/PBS for 2 hours at room temperature. Plates were washed twice with wash buffer and then twice in PBS. A 50-μL solution of BCIP/NBT (FASTR B-5655; Sigma Chemical Co.) was added for 20 minutes. The plates were then dried and counted.

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**Table 1**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Control</th>
<th>Bcl-xL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incidence</td>
<td>23/26</td>
<td>26/26</td>
</tr>
<tr>
<td>Mean maximal grade ± SEM</td>
<td>1.5 ± 0.3</td>
<td>2.2 ± 0.3^b</td>
</tr>
<tr>
<td>Day of onset ± SEM</td>
<td>15.7 ± 0.9</td>
<td>12.6 ± 0.6^c</td>
</tr>
<tr>
<td>Proportion with chronic EAE^d</td>
<td>11/26</td>
<td>18/26^e</td>
</tr>
<tr>
<td>Deaths</td>
<td>1/26</td>
<td>5/26</td>
</tr>
</tbody>
</table>

^aComposite data from 4 experiments (n = 26 per group). ^bP < 0.03 by Mann-Whitney U test. ^cP < 0.006 by Mann-Whitney U test. ^dChronic phase was considered to start on day 25; this is the number of animals with a higher grade than 1 after the acute phase. ^eP < 0.046 by Fisher’s exact test.

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**Figure 4**

Microphotograph (magnification ×200) of representative spinal cord sections from wild-type and Bcl-xL transgenic mice (n = 4 per group) after immunization with 200 μg of MOG p35–55. The spinal cords were collected when mice reached grade 2. The sections were immunohistochemically stained for activated macrophages (Mac-1), B220^+^ B cells (B220), and αβ TCR^+^ cells. Cells positive for the marker indicated display a brown color.
of labeled cells per 100 mm² of spinal cord tissue. Evaluation, and the results were expressed as mean number was evaluated for a given cellular marker at whole tissue section (a longitudinal spinal cord section) evaluated at least at 3 different levels of sectioning. The antibody served as negative controls. Each specimen was treated with 0.1% of Triton X-100 (Sigma Chemical Co.). Isotype-matched Ig and omission of the primary antibody were used. The sections were stained using the avidin-biotin technique (Vectastain Elite kit; Vector Laboratories), and then counterstained in hematoxylin. Isotype-matched IgG were purchased from Vector Laboratories (Burlingame, California, USA).

**Antibodies.** The following antibodies were obtained from PharMingen: purified rat IgG1, IgG2a, and IgG2b, purified hamster IgG, rat anti-mouse CD11b, rat anti-mouse CD45R/B220, rat anti-mouse IFN-γ/R4-6A2, rat anti-mouse IL4/BVD4-1D11, rat anti-mouse IL-10/JES5-2A5, rat anti-mouse IL-12/CI7.8, rat anti-mouse TNF-α/MP6XT22, and rat anti-mouse macrophage clone F4/80 (Caltag Laboratories Inc.). Biotinylated swine anti-rabbit Ig was obtained from DAKO A/S (Glostrup, Denmark), and biotinylated anti-hamster IgG and biotinylated anti-rat IgG were purchased from Vector Laboratories (Burlingame, California, USA).

**Immunohistology.** Spinal cords and brains were collected at the peak of disease (as mice reach grade 2) from 4 to 8 mice in each experimental group. Spinal cord tissues were embedded in OCT Compound (Tissue-Tek, Sakura Finetek, Torrance, California, USA), quick frozen in liquid nitrogen, and kept at −70°C until sectioning. Cryostat sections (10 μm) of spinal cords were fixed with acetone or 4% paraformaldehyde and then labeled with the antibody of interest. The sections were stained using the avidin-biotin technique (Vectastain Elite kit; Vector Laboratories), visualized with diamino-benzidine (Vector Laboratories), and then counterstained in hematoxylin. Isotype-matched Ig and omission of the primary antibody served as negative controls. Each specimen was evaluated at least at 3 different levels of sectioning. The whole tissue section (a longitudinal spinal cord section) was evaluated for a given cellular marker at ×40 magnification, and the results were expressed as mean number of labeled cells per 100 mm² of spinal cord tissue. Evaluation of cytokine expression was done semiquantitatively because of the tendency of cytokines to diffuse around cells. The following arbitrary scale was used: –, no positive cells; +, 1–9 positive cells; ++, 10–99 positive cells; +++, 100–1,000 positive cells; and ++++, if positive cells exceeded 1,000 per 100 mm² of tissue examined.

To assess demyelination, spinal cords were collected on days 10 and 13 after immunization, and quick-frozen sections were stained with the Luxol fast blue method. The sizes of demyelinating areas were measured throughout the sections by using KS 400 image analysis system (Carl Zeiss). The x axis represents the number of cells per 100 mm² of tissue. There were significant differences between the 2 groups in all the markers listed (AP < 0.0001, B P = 0.0004, CP = 0.0028 for B220 by t test).

**Results**

**Bcl-xL transgenic mice have a more severe disease induced by MOG peptide.** Bcl-xL transgenic mice have a more severe form of EAE after active immunization with MOG p35–55 than wild-type animals. Table 1 shows the composite data of 4 experiments. There was a significant difference in the mean maximal grade (P = 0.03) and the day of onset (P = 0.006) between the 2 groups, both of

<table>
<thead>
<tr>
<th>Groups</th>
<th>Area of demyelination (mm²)/100 mm² of spinal cord</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 10 after immunization</td>
<td>Day 13 after immunization</td>
</tr>
<tr>
<td>Wild-type</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>Bcl-xL</td>
<td>15.2 ± 1.4 A</td>
</tr>
</tbody>
</table>

Mice were immunized with MOG p35–55. One group of mice was sacrificed on day 10 and another on day 13 after immunization; the spinal cords were dissected and examined. Demyelination was assessed quantitatively at 3 different levels of sectioning. The numbers depicted here show mean values of demyelinated areas (mm²) per 100 mm² of tissue per each group (± SE). AP < 0.0001 by Mann-Whitney U test.
these measures reflect differences in the acute disease. Interestingly, there was also a difference in the chronic phase of the disease: in Bcl-xL transgenic animals, the proportion of mice with chronic disease (mice with grade 1 or more) was significantly higher (P = 0.04).

Susceptibility of Bcl-xL transgenic mice to develop severe EAE is not due to a broader autoactive T-cell repertoire. C57BL/6 mice are highly resistant to EAE induction by other myelin antigens such as MBP and PLP. To address the question of whether the expression of the Bcl-xL transgene renders mice susceptible to disease induced by other autoantigens, we immunized both Bcl-xL transgenic and wild-type mice with MBP or PLP and checked for EAE development. Both Bcl-xL transgenic mice and wild-type mice developed very mild EAE after MBP or PLP immunization, which was manifested by slight tail weakness. These results indicate that transgenic expression of the long form of the bcl-x gene targeted to the T-cell lineage does not alter the T-cell repertoire or broaden the pool of T cells capable of mediating EAE. After staining naïve splenic T cells or splenocytes from MOG-immunized mice with a panel of antibodies against Vβ chain of TCR, we found no significant differences in Vβ usage between Bcl-xL transgenic and wild-type littermates (Figure 1).

Bcl-xL transgenic mice have increased T-cell responses and improved survival after MOG p35–55 stimulation in vitro. As shown in Figure 2a, there was a significant increase in the in vitro proliferation (P = 0.04 for MOG 10 μg/mL and P = 0.002 for MOG 100 μg/mL) in draining lymph node cultures from Bcl-xL transgenic animals as compared with wild-type mice immunized with MOG p35–55. The precursor frequency of cells reacting to MOG was estimated by ELISPOT with the number of IFN-γ-secreting cells in the cultures from Bcl-xL mice, reaching significance at the highest antigen dose (P = 0.03). Figure 2b shows the number of MOG-reactive cells per 10⁵ cells plated. The number of cells decreased proportionately as the number of cells plated decreased, thus confirming the specificity of the response. There was no difference in Con A–stimulated IFN-γ-producing cells between wild-type and transgenic animals (not shown). Cytokine secretion was also significantly increased during in vitro stimulation with antigen (Figure 2, c and d), and cytokine secretion after Con A stimulation was similar between the 2 groups (not shown).

To determine if cells from Bcl-xL transgenic mice had improved survival in vitro, we used a TUNEL-staining method for flow cytometry. Primed lymph node cells were cultured in vitro with MOG p35–55, and the percentage of apoptotic cells in the culture was determined at various time points. As seen in Figure 3, the percentage of apoptotic cells (TUNEL-positive cells) was persistently higher in the control cultures when compared with the cultures from Bcl-xL transgenic mice (P = 0.0081 by repeated measures ANOVA); this was similar to our findings with antigen nonspecific stimulation (17). These findings were confirmed by measuring the percentage of 7AADlo cells in the cultures, because 7AADlo population stains apoptotic cells, whereas 7AADhi stains dead cells (22).

Bcl-xL transgenic mice have more extensive CNS inflammation and demyelination. There were marked differences in the histopathologic manifestation of EAE in the CNS of Bcl-xL transgenic mice when compared with wild-type mice, which were more prominent than the differences in clinical disease. Bcl-xL transgenic mice exhibited extensive demyelination on day 10 after immunization in both the spinal cord and brain, whereas wild-type mice showed no evidence of demyelination on day 10. On day 13 after immunization, both groups of mice had areas of demyelination, but it was more extensive in the CNS of Bcl-xL compared with wild-type mice (Table 2). The sections from Bcl-xL mice showed 64 ± 9 mm² of demyelination per 100 mm² of tissue compared with 15 ± 4 mm² in the wild-type CNS. Furthermore, Bcl-xL transgenic sections showed massive inflammation and inflammatory infiltrates consisting of activated macrophages/microglia (Mac-1⁺), B cells, and activated T cells (CD25⁺) (Figures 4 and 5). Furthermore, investigation of cytokine production in the CNS showed that Bcl-xL transgenic mice had more IFN-γ, IL-12, and TNF-α production in the CNS when compared with wild-type mice (Table 3). There were no significant differences in IL-4 and IL-10 in the CNS.

Bcl-xL transgenic mice have less apoptotic cells in the infiltrates. To investigate whether protection from apoptosis contributed to the worsening of EAE, we compared the number of apoptotic cells in the CNS of transgenic mice and littermates. TUNEL staining of spinal cord tissues was performed on samples taken on days 13–15, 20, and 50. At all time points tested there was a...
decreased number of apoptotic cells in the CNS infiltrates of Bcl-xL transgenic mice compared with wild-type mice. Bcl-xL transgenic mice had only occasional cells staining by TUNEL compared with the wild-type mice. Quantitation of the TUNEL-positive cells in the CNS showed significantly less apoptotic cells in the transgenic mice at all time points (Figure 6). Interestingly, the number of apoptotic cells in the wild-type control animals increased significantly between days 13 and 50 (P = 0.0001), whereas there was no change in the number of apoptotic cells in the Bcl-xL mice, suggesting a link between apoptosis and recovery from disease.

Discussion
Bcl-2 and related proteins such as Bcl-xL are products of a family of genes that modulate programmed cell death or apoptosis. The function of these genes is critical not only during development and tissue homeostasis, but also in the pathogenesis of a variety of diseases including autoimmune diseases, neurodegenerative disorders, cancer, and viral infections. A failure to undergo apoptosis or programmed cell death may contribute to the pathogenesis of these diseases. In EAE, clinical remissions and recovery from clinical symptoms may be related to inflammatory cell death through apoptosis. The role of activation-induced cell death though the Fas/FasL pathway has been investigated in EAE (11, 12), but the role of passive cell death is unknown. Here we address the role of T-cell apoptosis by passive cell death in EAE. We used mice with transgenic expression of the long form of the bcl-x gene targeted to the T-cell lineage (3, 4). In these mice, Bcl-xL is constitutively expressed in T cells and enhances their survival when cultured in vitro. We found that mice transgenic for Bcl-xL have an earlier onset and a more severe form of EAE induced by MOG p35–55 when compared with wild-type C57BL/6 mice and nontransgenic littermates.

The differences in pathology between transgenic and wild-type animals were even more prominent than the differences in clinical disease scores. This may reflect increased survival of the activated cells in the CNS, thus leading to a persistent chronic disease in the transgenic mice. The persistent T cells could also be providing more help for B cells even though the transgene is expressed in T cells. This is consistent with the increased demyelination seen in the Bcl-xL mice. Alternatively, the increased severity of EAE in Bcl-xL transgenic mice could be the result of broader autoreactive T-cell repertoire. Although it was shown that the bcl-xL gene does not prevent clonal deletion of autoreactive thymocytes (3), we questioned whether peripheral mechanisms of tolerance to self-reactive thymocytes that escaped central selection were disrupted by the transgenic expression of Bcl-xL. Thus, we immunized Bcl-xL transgenic and wild-type mice with other myelin antigens, MBP and PLP, and found that immunization with either of these did not induce significant clinical EAE in Bcl-xL transgenic or wild-type mice. Thus, the increased severity of EAE in Bcl-xL transgenic mice is likely due to increased survival of MOG-reactive T cells and/or to an increased capacity to proliferate and produce inflammatory cytokines. Hence, we studied the capacity of Bcl-xL transgenic T cells to proliferate and investigated their cytokine production.

Previous studies from our group with nonantigen specific stimulation, showed that Bcl-xL transgenic cells had fewer cell divisions than non-transgenic cells (17). However, our in vitro studies showed increased proliferation and cytokine production of peripheral antigen-specific T cells from Bcl-xL transgenic mice when compared with wild-type mice in response to MOG p35–55. This would suggest that the precursor frequency of MOG-specific T cells is higher in the lymph node cells from Bcl-xL transgenic mice, probably the result of enhanced survival of these cells after priming. Consistent with this data, we found an increased number of MOG-specific IFN-γ-secreting cells and enhanced survival of primed lymph node cells in vitro after stimulation with MOG peptide.

The clinical manifestations of disease correlated with increased inflammatory infiltrates in the CNS and increased local production of IFN-γ and TNF-α. Furthermore, there were more areas of demyelination in the Bcl-xL transgenic mice suggesting that the increased infiltrates translate to worsening pathology. TUNEL staining of the CNS during the peak of EAE showed less apoptotic cells in the transgenic mice compared with littermates. Furthermore, in the wild-type animals the number of apoptotic cells increased during the course of the disease, which is consistent with previous reports (23–25). Previous studies in the Lewis rat model reveal that during spontaneous recovery from EAE autoreactive T cells are eliminated from the CNS by activation-induced apoptosis involving the Fas pathway (23, 24). There are several pathways leading to elimination of T cells by apoptosis. Fas-mediated apoptosis may be crucial in the Lewis rat model, whereas passive cell death may be more important in the murine model, as shown by our data. In transplantation tolerance, both passive cell death and AICD appear crucial for tolerance induction (17). These results underscore the crucial role of T-cell apoptosis in the outcome of an autoimmune attack and indicate that termination of a T cell–mediated autoimmune reaction is dependent on clearance of autoreactive cells through programmed cell death. Infiltrating CD4+ T cells and parenchymal microglia express

### Table 3

Cytokine expression in the CNS at the peak of disease

<table>
<thead>
<tr>
<th></th>
<th>IFN-γ</th>
<th>TNF-α</th>
<th>IL-10</th>
<th>IL-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Bcl-xL+/+</td>
<td>++</td>
<td>+++</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Semiquantitative assessment of immunohistochemical staining for IFN-γ, TNF-α, IL-10, and IL-4 around the cellular infiltrates in spinal cord tissues from wild-type and Bcl-xL transgenic animals after immunization with MOG p35–55. The tissues were collected at the peak of clinical EAE on day 13 after immunization. Positive staining was measured semiquantitatively as follows: +, 1–10 positive cells; ++, 11–100 positive cells; +++, more than 100 positive cells.
Fas, FasL, and Bax in CNS, and the upregulation of these molecules is associated with disease activity (26). The role of Fas/FasL pathway in regulation of autoimmune responses remains controversial. Apoptosis mediated by Fas/FasL interaction was thought to be critical in development of EAE because mice with mutations in Fas or FasL expression are resistant to EAE (11, 12). More recently, studies in Fas-deficient SJL (SJL lpr/lpr) mice showed that even though the incidence of the disease is lower than that in heterozygous littermates, the disease was more severe and less likely to remit (13). The studies in these Fas/FasL-deficient models are complicated by the fact that the mutation is not restricted to the T-cell lineage, and apoptosis of microglia cells and possibly oligodendrocytes is also impaired. Fas may play an important role in mediating autoimmune tissue destruction. Upregulation of FasL on activated autoreactive T cells, together with upregulation of Fas on resident cells in the target organ may lead to direct target destruction. Thus, the absence of programmed cell death of microglial and oligodendrocyte may contribute to the resistance to EAE in these models.

By using a system where the antiapoptotic gene (Bcl-xL) is expressed exclusively on T cells, we could address the role of T-cell apoptosis in termination of neuroinflammation. We conclude that the survival of autoreactive T cells expressing the bcl-xL gene plays a critical role in the pathogenesis of EAE. These findings have implications for understanding the pathogenesis of multiple sclerosis as well as for other autoimmune diseases.

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