ARTHRITIS & RHEUMATISM
Vol. 46, No. 2, February 2002, pp 514–521
DOI 10.1002/art.10128

Published by Wiley-Liss, Inc.

Constitutive Expression of Bcl-x_L in the T Lineage Attenuates Collagen-Induced Arthritis in Bcl-x_L Transgenic Mice

Ying Chen,[1] Ed Rosloniec,[2] Jim Price,[3] Mark Boothby,[3] and Jin Chen[3]

Objective. **To determine if inhibition of T cell apoptosis through constitutive expression of Bcl-x_L in the T lineage influences inflammatory arthritis in the mouse collagen-induced arthritis (CIA) model.**

Methods. **The incidence and severity of arthritis were quantified in Bcl-x_L transgenic mice and nontransgenic littermates after immunization with type II collagen (CII). To correlate T cell responses with disease phenotype, antigen-specific T cell proliferation was measured by ^{3}H-thymidine incorporation. Apoptosis and cell cycle progression were analyzed by flow cytometry using propidium iodide. Production of CII-specific interferon-γ (IFNγ), interleukin-5 (IL-5), and IL-10 was determined by enzyme-linked immunosorbent assay.**

Results. **Disease severity in CIA was significantly attenuated in Bcl-x_L transgenic mice compared with their nontransgenic littermates. Inhibition of CIA was associated with decreased T cell apoptosis, delayed cell cycle progression, and reduced IFNγ production.**

Conclusion. **Rather than promoting inflammation, inhibition of apoptosis by expression of the Bcl-x_L protein in the T lineage attenuates disease progression in CIA, probably through inhibition of IFNγ production.**

Supported by the core facilities of the Vanderbilt-Ingram Cancer Center and Diabetes Research and Training Center. Dr. J. Chen's work was supported by NIH grant R01-HD-36400, American Heart Association grant 97300889N, ACS institutional research grant IN-25-38, and a grant from Boehringer Ingelheim Pharmaceutical Inc. Dr. Boothby's work was supported by a grant from Boehringer Ingelheim Pharmaceutical Inc., and NIH grants R01- GM-42550 and R01-HL-61752.

[1]Ying Chen, MD: Vanderbilt University Medical School, Nashville, Tennessee; [2]Ed Rosloniec, PhD: University of Tennessee, and the Research Service of the Veterans Administration Medical Center, Memphis, Tennessee; [3]Jim Price, PhD, Mark Boothby, MD, PhD, Jin Chen, MD, PhD: Vanderbilt-Ingram Cancer Center, and Vanderbilt University Medical School, Nashville, Tennessee.

Address correspondence and reprint requests to Jin Chen, MD, PhD, Vanderbilt University School of Medicine, A-4323 MCN, 1161 21st Avenue South, Nashville, TN 37232. E-mail: jin.chen@mcmail.vanderbilt.edu.

Submitted for publication June 18, 2001; accepted in revised form September 24, 2001.

Effector T cells play a critical role in controlling pathologic immune responses, including infections and allergic and autoimmune disorders. Consequently, homeostasis of effector T lymphocytes is tightly regulated through the balance between cell proliferation and apoptotic cell death. Resistance to cell death has been implicated in autoimmune diseases such as diabetes mellitus (1) and experimental autoimmune encephalomyelitis (EAE) (2). In rheumatoid arthritis (RA), apoptosis was detected in macrophages and fibroblasts, but not in T lymphocyte aggregates, in patient synovium (3,4). In addition, RA patients have a subset of CD4+,CD28− T cells in the peripheral blood that frequently undergoes clonal expansion and is resistant to apoptosis (5). Taken together, these data suggest that altered apoptotic responses in T cells might play a critical role in the pathogenesis of RA.

Molecular mechanisms of dysregulated apoptosis in T lymphocytes in arthritis have been investigated. Changes in apoptotic or antiapoptotic protein levels have been correlated with the disease pathogenesis. One example is the Fas protein, a member of the tumor necrosis factor receptor family that is critical for inducing cell death. Synovial T lymphocytes exhibit decreased levels of Fas ligand (6), resulting in ineffective clearance of activated T cells. Furthermore, soluble Fas is accumulated in inflamed joints of RA patients, suggesting that soluble Fas may inhibit Fas ligand–mediated apoptosis of infiltrating T lymphocytes (7).

A second family of molecules that regulates apoptosis is the nuclear factor κB (NF-κB)/Rel transcription factors. The NF-κB transcription factor family is activated in T cells after engagement of the T cell receptor, costimulatory molecules, and cytokine receptors. A dominant negative inhibitor of nuclear factor κBα (IκBα) mutant (IκBα[ΔN]) constitutively inhibits NF-κB activation, and T cell–specific expression of this mutant renders transgenic mice resistant to the development of collagen-induced arthritis (CIA) (8). The

inhibition of CIA is accompanied by increased susceptibility to apoptosis in peripheral T cells (9), suggesting that increased apoptosis in transgenic T cells may contribute to the disease phenotype. A third gene family, the Bcl-2 superfamily, also plays a central role in regulating apoptotic cell death. Bcl-2 and related antiapoptotic proteins are up-regulated in the T cells of RA patients (4,5), indicating that elevated Bcl-2 and Bcl-x_L levels favor the clonal outgrowth of autoreactive T cells and contribute to the pathogenesis of RA.

To investigate the effect of altered apoptotic susceptibility on arthritis in vivo, we used transgenic mice that constitutively express Bcl-x_L specifically in the T lineage (10). In nontransgenic mice, Bcl-x_L is barely detectable in resting T cells, but is induced upon T cell activation (11). In Bcl-x_L transgenic mice, Bcl-x_L is constitutively expressed. Thymocytes from Bcl-x_L transgenic mice are resistant to apoptosis induced by glucocorticoid, irradiation, and anti-CD3 stimulation (10,12). Constitutive expression of Bcl-x_L in peripheral T cells also protects splenocytes and lymph node cells from programmed cell death (Mora AL, et al: unpublished observations). When EAE is induced in one of the Bcl-x_L transgenic lines (12), Bcl-x_L transgenic mice exhibit an earlier onset and a more severe form of the disease (13), and this phenotype is associated with increased proliferation and cytokine production in peripheral lymphocytes. These data suggest that the survival of autoreactive T cells expressing the Bcl-x_L gene plays a critical role in the pathogenesis of EAE, and may also be important in other autoimmune diseases such as RA.

In this study we found that, contrary to an expectation based on results with EAE, CIA is less severe in Bcl-x_L transgenic mice than in nontransgenic littermates. Inhibition of CIA was associated with resistance of T cells to apoptosis, delayed cell cycle progression, and marked attenuation of interferon-γ (IFNγ) production in response to type II collagen (CII) challenge, the net effect of which was to lessen the severity of arthritis.

MATERIALS AND METHODS

Mice. DBA/1 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and used at 7–8 weeks of age. Bcl-x_L transgenic mice were generously provided by Dr. Craig B. Thompson (10). To introduce CIA susceptibility genes (the H-2^q haplotype and other background genes), Bcl-x_L transgenic mice were crossed with DBA/1 for 2 generations (F_1N_1). Mice were then screened for Bcl-x_L and H-2 by Southern blot analysis and polymerase chain reaction (PCR), respectively. The primer set 5′-GATACAGCTGGAGTCAGTTTA-3′ and 5′-GTAGCCATTGCAGCTAGGTG-3′ was used to amplify a 700-bp product of the Bcl-x_L gene. In addition, the primer set 5′-ACCAACGGGACGCAGCGCAT-3′ and 5′-CCTCGTAGTTGTGTCTGCAC-3′ was used to amplify the 200-bp product of the I-Aβ gene. The PCR products were then probed with an oligonucleotide primer specific for H-2^q, H-2^b, or H-2^d genes. Primers 5′-ATACGATCTGTGAACAGATA-3′, 5′-ATACGATATGTGACCAGATA-3′, and 5′-ATACGGCTCGTGACCAGATA-3′ were specific for H-2^q, H-2^b, or H-2^d genes, respectively. Bcl-x_L transgenic mice homozygous for H-2^q were then further backcrossed to DBA/1 for 6 additional generations (F_1N_7). Collagen-specific Vβ8.3 transgenic mice were provided by one of the authors (ER) (14). Primer set 5′-CTCTTCTAGAACACATGGAGG-3′ and 5′-GACAGACAGCTTGGTTCCATG-3′ was used to amplify a 346-bp product of the Vβ8.3 gene. IκBα(ΔN) transgenic mice have been described previously (8).

Western blot analysis. Single-cell suspensions from the thymus, spleen, and lymph node were prepared by crushing the organs in complete media (RPMI 1640 supplemented with 10% fetal calf serum, 50 μM β-mercaptoethanol [β-ME], 2 mM L-glutamine, and 0.1% penicillin-streptomycin), followed by hypotonic lysis of erythrocytes. Splenocytes were depleted of T cells by incubating with anti–Thy-1 antibody for 30 minutes at 4°C, followed by washing and subsequent incubation with rabbit complement for 45 minutes at 37°C. More than 95% of T cells were depleted following this procedure, as assessed by flow cytometry. T cell–depleted splenocytes were lysed using RIPA buffer (1× phosphate buffered saline [PBS], 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS]) with protease inhibitors (100 μg/ml phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 45 μg/ml aprotinin). Thirty-microgram proteins were fractionated by SDS–polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and blotted with 250 ng/ml anti-mouse Bcl-x_L polyclonal antibody (Transduction Laboratories, San Diego, CA) and anti-mouse β-tubulin polyclonal antibody (1:500; Sigma, St. Louis, MO). Immunoreactive polypeptides were then detected with anti-rabbit IgG (for Bcl-x_L) and anti-mouse IgG (for β-tubulin) conjugated to horseradish peroxidase, using enhanced chemoluminescence (Amersham, Arlington Heights, IL).

Induction and assessment of CIA. Native bovine CII (Chondrex, Seattle, WA) was dissolved at 2 mg/ml in 0.01M acetic acid at 4°C overnight, and emulsified with an equal volume of complete Freund's adjuvant (CFA) (Difco, Detroit, MI). CIA was induced by intradermal injection at the base of the tail with 100 μl of emulsion containing 100 μg of CII; 21 days after the primary immunization, mice were boosted with 0.1 ml of emulsion containing 100 μg of CII in incomplete Freund's adjuvant (IFA). Mice were monitored in a blinded manner, by 2 independent examiners, for signs of arthritis and date of disease onset. Clinical arthritis was assessed by the following standard scoring system: grade 0 = no swelling; grade 1 = paws with swelling in a single digit; grade 2 = paws with swelling in multiple digits; grade 3 = severe swelling and joint rigidity. Each limb was graded, giving a maximum possible score of 12 per mouse. Data were analyzed using the Macintosh InStat software program. Group comparisons were performed using the chi-square test for disease incidence and Student's unpaired 2-tailed t-test for arthritis scores.

Proliferation assay. Mice were killed on day 10 after immunization. Draining lymph nodes (inguinal, paraaortic) were excised, and single-cell suspensions were prepared in serum-free HL-1 medium (BioWhittaker, Walkersville, MD) supplemented with L-glutamine, β-ME, and antibiotics. T cells were purified with a nylon wool column, and the resulting T cell purity was >90% as assessed by flow cytometry. Irradiated splenocytes obtained from syngeneic nontransgenic littermates were used as antigen-presenting cells (APCs). Purified T cells (4.0×10^5 cells/well) were plated with APCs (1.0×10^5 cells/well) in triplicate in 96-well flat-bottomed microtiter plates in medium alone or in the presence of CII synthetic peptide at 3.3 and 33 μg/ml (ATGPLGPKGQTGEBGIAG-FKGEQGPK; a generous gift from Dr. D. D. Brand, University of Tennessee, Memphis). Cells were incubated at 37°C in 5% CO_2 for 4 days, and 1 μCi/well of ^{3}H-thymidine (^{3}H-TdR) was added in culture for the last 18 hours. Cells were harvested, and ^{3}H-TdR incorporation per well was measured using a beta scintillation counter (Beckman Instruments, Irvine, CA).

Cytokine assays. Draining lymph nodes were removed 2 and 6 weeks after immunization. Single-cell suspensions were prepared and cultured in RPMI 1640 containing 10% fetal bovine serum. The cells were cultured in 96-well round-bottomed plates for 72 hours at 2×10^6 cells/ml (200 μl/well) in medium alone, or with 5 or 50 μg/ml of heat-denatured CII. Supernatants were harvested and analyzed for IFNγ, interleukin-5 (IL-5), and IL-10 by sandwich enzyme-linked immunosorbent assay using antibody pairs (PharMingen, San Diego, CA), according to the manufacturer's recommended procedures.

DNA content/cell cycle distribution analysis. At least 1×10^6 draining lymph node cells were collected and stained with the fluorescein isothiocyanate–conjugated anti-CD3 antibody (PharMingen). The cells were washed with PBS, fixed in 1 ml ice-cold 70% ethanol, and stored at −20°C overnight in the dark. The ethanol was removed by washing with PBS, and the cells were incubated in 500 μl propidium iodide (0.1 m*M* EDTA, 0.02 mg/ml propidium iodide, 0.1% Triton X-100 in PBS; Sigma) for 30 minutes at room temperature in the dark. Flow cytometric analysis of DNA content was then performed using a FACScan (Becton Dickinson, Mountain View, CA). Cell cycle analysis was conducted using "ModFit" cell cycle software and "WinList" histogram software (Verity Software House, Topsham, ME). Apoptotic cell death was quantified as the proportion of cells in the population with a subdiploid (<2N) DNA content.

RESULTS

Specific expression of Bcl-x_L transgene in the T lineage of adult lymphoid organs. To determine if the Bcl-x_L transgene would exert a direct effect only on T cells, we examined whether Bcl-x_L proteins were expressed in adult peripheral lymphoid organs and whether the expression of the transgene was restricted to the T lineage. As shown in Figure 1, the Bcl-x_L protein was expressed at high levels in spleens and lymph nodes from transgenic mice that were 7 or 8 weeks old (Figure 1A, lanes 2, 3, 5, and 6) compared with nontransgenic littermates (Figure 1A, lanes 1 and 4). To determine whether Bcl-x_L expression was restricted in the T lineage, splenocytes were depleted of T cells by Thy-1–mediated complement lysis. As shown in Figure 1B, while Bcl-x_L was expressed in the thymus, lymph nodes, and spleen (lanes 1–3), Bcl-x_L protein was not detectable in T cell–depleted splenocytes (lane 4). Taken together, these results indicated that the expression of the Bcl-x_L transgene was persistently high in adult peripheral lymphoid organs in transgenic animals and expression was specifically restricted to the T lineage, thus providing a model to evaluate the effect of suppressing apoptosis specifically in T lineage cells during CIA pathogenesis.

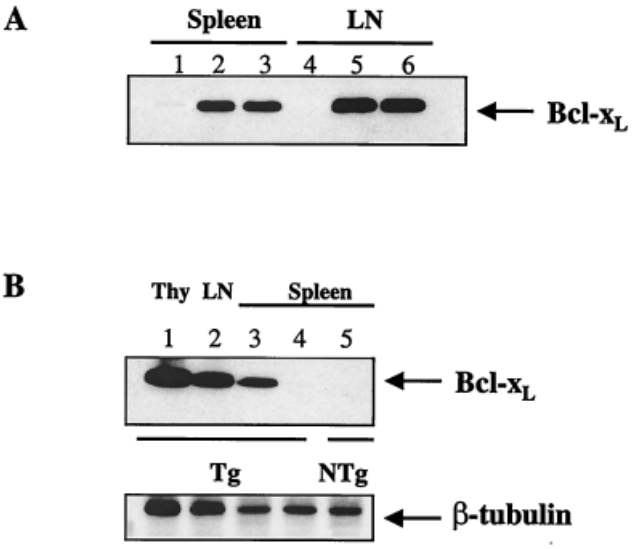

Figure 1. Expression of Bcl-x_L transgene in the T lineage of adult peripheral lymphoid organs. **A,** Expression of Bcl-x_L transgene in spleen and lymph nodes (LN). Splenocyte and lymph node cell lysates were prepared from 7–8-week-old animals and analyzed by Western blot using an anti–Bcl-x_L antibody. Lanes 2, 3, 5, and 6, Transgenic mice; lanes 1 and 4, nontransgenic littermates. **B,** Expression of Bcl-x_L transgene is restricted to the T lineage. Transgenic splenocytes were depleted of T cells by anti-Thy-1–mediated complement lysis (lane 4). Cell lysates prepared from transgenic mice (Tg) and nontransgenic littermates (NTg) were analyzed by Western blot, as described in **A.** Lane 1, Thymocyte (Thy); lane 2, lymph node cells; lanes 3–5, splenocyte; lane 4, T cell–depleted splenocyte. The same membrane was stripped and reprobed with anti–β-tubulin antibody as an internal control for equal loading. Note that the β-tubulin content is different in the various organs, such as the thymus, lymph nodes, and spleen, although the total loading amount of protein from these organs is the same.

Reduced severity of CIA in Bcl-x_L transgenic mice. We have previously reported that inhibition of NF-κB in the T lineage suppressed the development and

progression of CIA in IκBα(ΔN) transgenic mice (8). The inhibition of CIA is accompanied by increased susceptibility to apoptosis in peripheral T cells (9). These findings suggest that increased apoptosis in IκBα(ΔN) T cells may contribute to the disease-resistant phenotype. Alternatively, it might be that enhanced deletion of collagen-specific T cells was not the critical factor, but rather that their effector function was impaired. To differentiate these two possibilities, we introduced the antiapoptotic gene Bcl-x_L into IκBα(ΔN) T cells. Recently, Mora et al showed that the NF-κB blockade inhibited endogenous Bcl-x_L induction upon T cell activation, and constitutive expression of Bcl-x_L in T cells protected cells from apoptosis (Mora AL, et al: unpublished observations). Thus, expression of Bcl-x_L in IκBα(ΔN) T cells would reverse the disease phenotype in IκBα(ΔN) transgenic mice. To test this hypothesis, IκBα(ΔN) and Bcl-x_L transgenic mice were bred to the DBA/1 genetic background and intercrossed to generate IκBα(ΔN)/Bcl-x_L double-transgenic mice. Since both transgenes are under the control of the lck promoter/enhancer, each is specifically expressed in the T lineage. The transgenic mice and nontransgenic littermates were immunized with 100 μg CII in CFA, boosted with 100 μg CII in IFA after 21 days, and monitored for the occurrence of clinical signs of arthritis.

Three separate experiments were conducted (Table 1). The results from these 3 experiments were pooled, and the incidence and the mean arthritis scores of all animals in each group were calculated. As shown in Table 1 and Figure 2, Bcl-x_L did not alter the disease-resistant phenotype in IκBα(ΔN) transgenic mice, as judged by disease incidence, severity, and date of onset. Since IκBα(ΔN) T cells exhibit multiple defects in T cell activation and effector functions (8,9), one of the possibilities is that increased apoptosis in IκBα(ΔN) T cells does not play a major role in CIA pathogenesis. Alternatively, Bcl-x_L may suppress CIA directly.

To directly determine the role of Bcl-x_L in arthritis, CIA was induced in Bcl-x_L transgenic mice and nontransgenic littermates under the same immunization protocol as described above. Pooled data from 3 separate experiments showed that constitutive expression of Bcl-x_L provided protection against inflammatory arthritis. As shown in Figure 2 and Table 2, the arthritis score was significantly decreased in Bcl-x_L transgenic mice (mean ± SEM) (4.2 ± 0.81) compared with that of wild-type littermates (7.6 ± 1.03) ($P < 0.05$). The incidence was reduced from 92.9% (13 of 14 mice) to 64.3% (9 of 14 mice). There was no discernible difference in the day of onset between the 2 groups (Table 2).

Table 1. Disease phenotype in dominant negative inhibitor of nuclear factor κBα transgenic (IκBα[ΔN]Tg) mice with and without Bcl-x_L *

Experiment/mice	Incidence, no.	Day of onset	Arthritis score, mean†
1/nontransgenic	1/1	26	12
1/IκBα(ΔN)Tg	3/4	35	1.75
1/IκBα(ΔN)/Bcl-x_LTg	2/4	33	0.75
2/nontransgenic	2/2	21	8.5
2/IκBα(ΔN)Tg	0/3	NA‡	0
2/IκBα(ΔN)/Bcl-x_LTg	0/2	NA	0
3/nontransgenic	2/2	35	5.5
3/IκBα(ΔN)Tg	1/5	35	0.6
3/IκBα(ΔN)/Bcl-x_LTg	0/4	NA	0
Total/nontransgenic	5/5	26	8 ± 1.8§
Total/IκBα(ΔN)Tg	4/12	35	0.83 ± 0.4§¶
Total/IκBα(ΔN)/Bcl-x_LTg	2/10	33	0.3 ± 0.2§¶

* Mice were immunized with 100 μg of type II collagen (CII) in complete Freund's adjuvant on day 1 and boosted on day 21 with 100 μg of CII in incomplete Freund's adjuvant.
† Clinical severity of arthritis in each joint was graded as follows: 0 = no swelling; 1 = paws with swelling in single digit; 2 = paws with swelling in multiple digits; 3 = severe swelling and joint rigidity. Each paw was graded, and the 4 scores were added so the maximum score per mouse was 12.
‡ NA = not applicable.
§ Mean ± SEM from 3 experiments.
¶ $P < 0.05$ versus nontransgenic littermates, by Student's *t*-test.

These results demonstrated that overexpression of Bcl-x_L in the T cells attenuated the development of CIA.

Delayed cell cycle entry and decreased apoptosis in Bcl-x_L transgenic mice. To elucidate the mechanism by which altered T cell function in Bcl-x_L transgenic mice led to alleviation of disease, we first tested collagen-specific proliferative responses of draining lymph node cells from Bcl-x_L transgenic mice and their nontransgenic littermates on a CII-specific Vβ8.3 T cell receptor transgenic background (15). T cells were isolated from Bcl-x_L/Vβ8.3 double-transgenic mice or their Vβ8.3 control littermates immunized with CII, and then stimulated with 1 or 10 μg/well of CII synthetic peptide or anti-CD3 antibody. As shown in Figure 3, T cells from both control and Bcl-x_L transgenic mice responded vigorously to CII peptide in a dose-dependent manner. Proliferation of Bcl-x_L transgenic peripheral T cells was markedly decreased compared with those of control littermates under stimulation by anti-CD3 antibody. However, Bcl-x_L transgenic mice and control littermates exhibited no significant difference in proliferation upon CII challenge.

Overexpression of Bcl-2 has been shown to reduce proliferation of T cells and delay cell cycle entry of polyclonal-stimulated T lymphocytes (16–18). To determine whether Bcl-x_L also affects cell cycle progression in

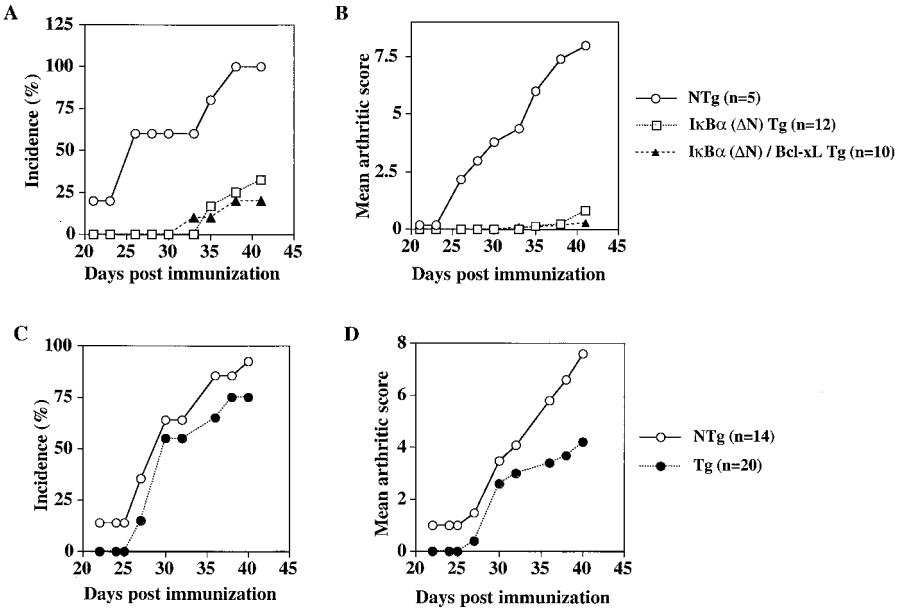

Figure 2. Reduced severity of collagen-induced arthritis (CIA) in Bcl-x_L transgenic (Tg) mice. Dominant negative inhibitor of nuclear factor κBα (IκBα[ΔN]), IκBα(ΔN)/Bcl-x_L transgenic mice **(A** and **B),** or Bcl-x_L transgenic mice and nontransgenic (NTg) control littermates **(C** and **D)** were immunized, and incidence and severity of arthritis in transgenic and nontransgenic littermates were scored as described in Table 1. Results were pooled from 3 separate experiments (shown in Tables 1 and 2) and expressed as a percentage of the value in arthritic mice **(A** and **C)** and as the mean arthritis scores of all mice in each group on a given day during the course of CIA **(B** and **D).**

CII-specific T cells, draining lymph node cells from Bcl-x_L/Vβ8.3 and Vβ8.3 control mice were isolated 10 days postimmunization, restimulated with CII at 50 μg/ml for 3 days, and dual-stained with anti-CD3 and propidium iodide to assess T cell DNA content. As shown in Figure 4A, Bcl-x_L significantly delayed the cell entry from the G0/G1 phase to the S phase. After priming with collagen, 71.9 ± 4.8% of cells (mean ± SEM) in Bcl-x_L transgenic mice were retained in the G0/G1 phase, compared with 47.3 ± 5.7% cells in

Table 2. Collagen-induced arthritis in Bcl-x_L transgenic mice*

Experiment/mice	Incidence, no.	Day of onset	Arthritis score, mean
1/nontransgenic	4/5	26	3.6
1/transgenic	3/6	28	1.5
2/nontransgenic	3/3	27	9.7
2/transgenic	6/8	27	3.5
3/nontransgenic	6/6	22	10
3/transgenic	6/6	26	7.7
Total/nontransgenic	13/14	26	7.6 ± 1.03†
Total/transgenic	9/14	27	4.2 ± 0.81†‡

* See Table 1 for explanations.
† Mean ± SEM from 3 experiments.
‡ $P < 0.05$ versus nontransgenic littermates, by Student's *t*-test.

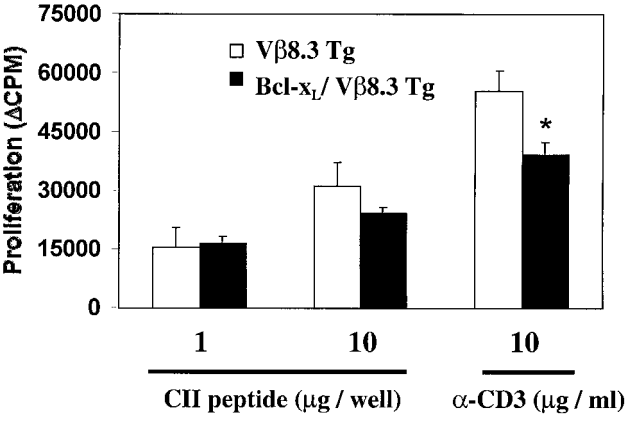

Figure 3. Antigen-specific T cell proliferation in Bcl-x_L transgenic (Tg) mice: proliferative response of T cells to type II collagen (CII) peptide. Draining lymph node cells from Bcl-x_L transgenic mice and nontransgenic littermates on a CII-specific T cell receptor Vβ8.3 transgenic background were isolated on day 10 after immunization, T cells were purified using a nylon wool column, and proliferation was measured in the presence of irradiated antigen-presenting cells in medium alone or in response to CII peptides. Values are the mean and SEM per group, as analyzed in 3 separate experiments. Background counts (counts per minute) for lymph node cells in Bcl-x_L transgenic mice and nontransgenic littermates were 3,142 ± 213 and 3,539 ± 724, respectively. * = $P < 0.05$ versus nontransgenic littermates, by Student's *t*-test.

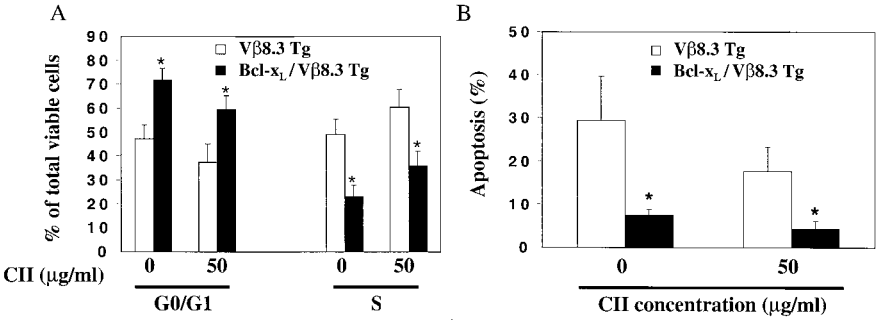

Figure 4. Delayed cell cycle entry and decreased apoptosis in Bcl-x_L transgenic (Tg) mice. Cell cycle distribution **(A)** and apoptosis **(B)** of lymph node cells from Bcl-x_L transgenic or control littermates. Draining lymph node cells from Bcl-x_L transgenic mice and nontransgenic littermates on type II collagen (CII)–specific T cell receptor Vβ8.3 transgenic background were isolated on day 10 after immunization and rechallenged with medium alone or CII. The DNA content of gated CD3+ T cells was analyzed by flow cytometry using propidium iodide. Values are the mean and SEM per group, as analyzed in 3 separate experiments. $* = P < 0.05$ versus nontransgenic littermates, by Student's *t*-test.

nontransgenic littermates under basal conditions ($P < 0.05$). There were proportionally more cells in the S phase in control mice than in transgenic littermates (49.2 ± 6.4% versus 23 ± 5%; $P < 0.05$). When cells were restimulated with CII, entry into the S phase for T lymphocytes from both transgenic and nontransgenic littermates was accelerated. However, there were significantly fewer cells in the S phase in Bcl-x_L transgenic mice than in control mice (36 ± 6.2% versus 60.4 ± 7.6%; $P < 0.05$). There was no difference in the G2/M phase between Bcl-x_L transgenic mice and control mice. Thus, this effect occurred predominantly at the transition from the G1 phase to the S phase, which is a crucial decision point between continued cell cycle progression or the induction of programmed cell death. When the survival of T cells was measured, apoptotic cell death was dramatically decreased in Bcl-x_L transgenic mice compared with nontransgenic littermates, both under basal and stimulated conditions (Figure 4B), indicating that constitutive expression of Bcl-x_L inhibits the apoptosis in peripheral T cells. Taken together, our findings suggest that Bcl-x_L delays the cell cycle progression of collagen-stimulated T cells and protects cells from apoptotic cell death.

Diminished IFNγ production in Bcl-x_L transgenic mice. Upon antigenic challenge, naive T cells proliferate and differentiate into Th1 and Th2 effector cells. A variety of data are consistent with the conclusion that the balance of cytokines produced by Th1/Th2 subsets of T cells plays an important role in the development of autoimmune disease.

To ascertain the impact of Bcl-x_L on the production of type 1 and type 2 cytokines, the levels of Th1 cytokine IFNγ and Th2 cytokines IL-10 and IL-5 were measured. Draining lymph node cells were isolated from Bcl-x_L transgenic mice and nontransgenic littermates 10 days and 6 weeks postimmunization, and restimulated with 0, 5, and 50 μg/ml CII. As shown in Figure 5A, the nontransgenic mice produced high levels of IFNγ upon stimulation with CII, whereas IFNγ production in transgenic mice exhibited a moderate decrease 10 days after immunization, and a more dramatic decline 6 weeks after immunization ($P < 0.05$). No significant differences in type 2 cytokine secretion (IL-5, IL-10) were detected 10 days and 6 weeks after immunization between Bcl-x_L transgenic mice and control littermates (Figures 5B and C). To assess whether Bcl-x_L could affect humoral responses to CII for the duration of arthritis, levels of anti-CII IgG1, IgG2a, and IgG were quantified from sera of Bcl-x_L transgenic mice and nontransgenic control littermates at 2 weeks, 4 weeks, and 6 weeks after immunization, and no significant differences were observed (data not shown). In summary, these results suggest that T cell–specific expression of Bcl-x_L suppresses type 1 cytokine production in response to antigenic challenge, providing a direct association between the diminished inflammatory cytokine production and alleviation of disease.

DISCUSSION

A balance between cell proliferation and cell death is essential for the normal function of the immune system. Resistance to apoptosis in peripheral blood T

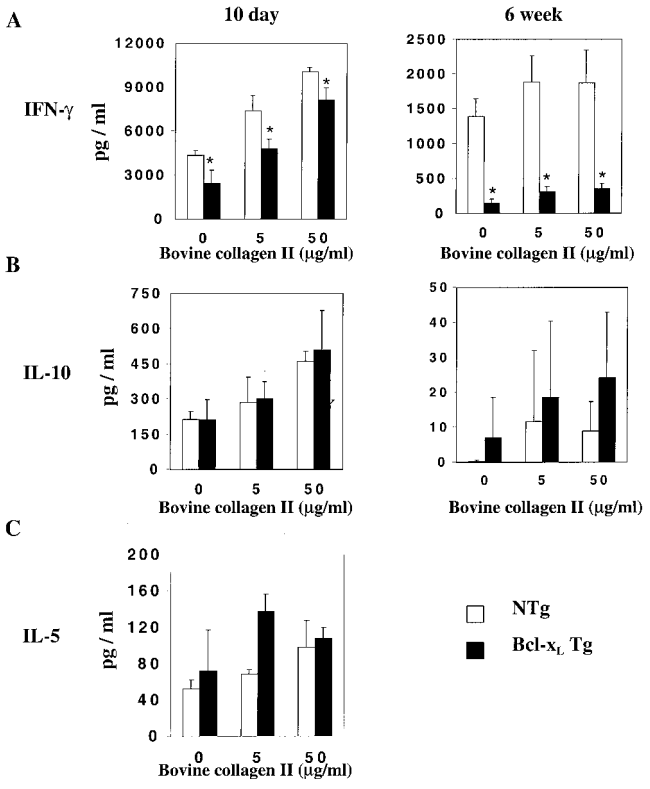

Figure 5. Diminished interferon-γ (IFNγ) and normal interleukin-10 (IL-10) and IL-5 production in Bcl-x_L transgenic (Tg) mice. Lymph node cell suspensions were prepared from draining lymph nodes from mice 10 days and 6 weeks after immunization with type II collagen (CII). Supernatant from cultures was collected after a 72-hour incubation in the presence of 0, 5, and 50 μg/ml of CII and assayed for IFNγ **(A),** IL-10 **(B),** and IL-5 **(C)** by enzyme-linked immunosorbent assay. Data are the mean and SEM, as analyzed in 2 separate experiments. $* = P < 0.05$ versus nontransgenic littermates, by Student's *t*-test. NTg = nontransgenic.

cells and synovial T cells has been suggested to play an important role in the pathogenesis of RA (3,4). Indeed, induced apoptosis of activated arthritogenic T lymphocytes has been considered as a potential therapeutic strategy (19). In principle, then, constitutive expression of antiapoptotic genes in T cells might prevent cells from death, thus exacerbating inflammatory arthritis. To test this hypothesis, we studied the development and progression of CIA in transgenic mice expressing Bcl-x_L specifically in the T lineage. Here we report that constitutive expression of Bcl-x_L in the T cells of transgenic mice substantially attenuates the severity of CIA, indicating an additional role of Bcl-x_L in CIA independent of its antiapoptotic function.

Based on our finding that Bcl-x_L delays the transition from the G1 phase to the S phase during cell cycle progression, it is conceivable that disruption of normal cell cycle regulation leads to defective T cell proliferation, thereby inhibiting disease severity in CIA. However, our available data do not support this notion. Although proliferative responses are reduced in Bcl-x_L–expressing T cells in response to anti-CD3 stimulation, Bcl-x_L T cells proliferate vigorously in response to antigen-specific challenge, and there is no significant difference in proliferation between Bcl-x_L–expressing cells and wild-type control T cells. In addition, since antibody production requires a T cell helper function, defects in T cell proliferation may lead to alterations in antibody levels. However, our data revealed that there was no significant change in CII-specific antibody levels.

An alternative mechanism is that alleviation of disease severity in CIA by Bcl-x_L is achieved by inhibition of inflammatory cytokine IFNγ production. IFNγ can promote disease by enhancing antigen presentation, by augmenting expression of major histocompatibility complex class II and cell adhesion molecules, or promoting Th1 cell differentiation and activation of macrophages (20). Although IFNγ perhaps has other contradictory immunoregulatory roles because inactivation of the IFNγ receptor accelerates CIA instead of attenuating disease (21), our data are consistent with the disease-promoting role of IFNγ in CIA. It is currently unclear how constitutive expression of Bcl-x_L leads to inhibition of IFNγ production in T lymphocytes. In endothelial cells, Bcl-x_L exerts an antiinflammatory function through inhibition of NF-κB (22), while in macrophages, Bcl-x_L inhibits lipopolysaccharide-induced cytokines through down-regulation of p38 mitogen-activated protein (MAP) kinase and c-Jun N-terminal kinase (JNK) repression (23). It remains to be determined whether inhibition of IFNγ production by Bcl-x_L in T cells is mediated through inhibition of NF-κB or down-regulation of p38 MAP kinase or JNK.

Our data seem somewhat unexpected, considering that a different line of transgenic mice expressing T lineage–specific Bcl-x_L was resistant to induction of transplantation tolerance (24) and exhibited worse EAE (13). It is possible that the contradictory findings reflect intrinsic differences between the role of T cells in CIA as compared with EAE. However, there are subtle differences between these 2 lines of Bcl-x_L transgenic mice. First, the Bcl-x_L transgene used in transplantation and EAE studies was under the control of the Eμ promoter/enhancer (12), while the Bcl-x_L transgene used in this study was under the control of the lck promoter (10). However, since both transgenes are targeted to the T

lineage, it seems unlikely that phenotypic differences in these 2 lines are due to differences in promoter/enhancer elements.

Second, Bcl-x_L transgenic mice used in studies on transplantation and EAE were on a C57BL/6 genetic background (H-2^b), while mice used in this study were on DBA/1 (H-2^q). It is possible that different genetic backgrounds may modify biologic responses to constitutive expression of Bcl-x_L. In the study on EAE, the levels of IFNγ increased dramatically in response to antigenic challenge in transgenic mice, while production of IFNγ was significantly decreased upon challenge with CII in our study.

Finally, it is conceivable that there are differences in the level of Bcl-x_L expression in the respective mice. In this regard, intracellular staining for the lck–Bcl-x_L transgene leads to protein levels comparable with the level of Bcl-x_L in wild-type T cells activated with anti-CD3/anti-CD28 (Mora AL, et al: unpublished observations). Thus, this transgene generates Bcl-x_L levels that are quite relevant to the normal activated T cell.

In summary, our data suggest that Bcl-x_L, independent of its antiapoptosis function, can inhibit the progression of CIA, probably through inhibition of inflammatory T cells and their production of cytokine IFNγ.

ACKNOWLEDGMENTS

We are grateful for excellent technical assistance from Erin Thompson; helpful discussions with Mark Aronica, Ana Mora, and David Brand (University of Tennessee, Memphis); a critical reading of the manuscript by Dana Brantley, James Thomas, Mark Aronica, and Ana Mora; and assistance with flow cytometry from Cathy Allen (Vanderbilt Cancer Center Flow Cytometry Core).

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