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Overexpression of Bcl\textsubscript{XL} in B Cells Promotes Th1 Response and Exacerbates Collagen-Induced Arthritis

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B cells play a pathogenic or regulatory role in many autoimmune diseases through production of autoantibodies, cytokine production, and Ag presentation. However, the mechanisms that regulate these B cell functions under different autoimmune settings remain unclear. In the current study, we found that when B cells overexpress an antiapoptotic gene, Bcl\textsubscript{XL}, they significantly increased production of IFN-\gamma and enhanced Th1 response. Consistently, Bcl\textsubscript{XL} transgenic mice developed more severe and sustained collagen-induced arthritis due to the enhanced Th1 response. The production of autoantibodies in Bcl\textsubscript{XL} transgenic mice was comparable to that in wild-type mice. Thus, our results indicate a novel role of Bcl\textsubscript{XL} in regulating B cell functions and immune responses. In patients with rheumatoid arthritis, arthritogenic B cells often up-regulate Bcl\textsubscript{XL} expression, which may not only render B cells resistant to apoptosis but also alter the ability of the autoreactive B cells to produce cytokines and modulate the inflammatory response. This may have therapeutic implications if Bcl\textsubscript{XL} expression can be down-regulated in autoreactive B cells.

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Bcl\textsubscript{XL} is an antiapoptotic molecule and belongs to the Bcl-2 family. The Bcl-2 and Bcl\textsubscript{XL} proteins enhance the survival of lymphocytes and other cell types. During B cell ontogeny, Bcl\textsubscript{XL} is expressed at highest levels in the small pre-B cell stage of development (1). Bcl\textsubscript{XL} expression is also detected in early pro-B and late pro-B/early pre-B cells but down-regulated in immature and mature B cells (1, 2). Although Bcl-2 plays a critical role in the survival of mature naive lymphocytes (3), Bcl-x\textsubscript{L} is important for the survival of immature lymphocytes (4). Significantly, Bcl\textsubscript{XL}, but not Bcl-2, is rapidly induced in peripheral B cells upon BCR crosslinking (2). In addition, Bcl-x\textsubscript{L} is up-regulated in germinal center (GC) B cells, suggesting Bcl-x\textsubscript{L} may play a critical role in regulating cell survival in the GC (5–7).

In rheumatoid arthritis (RA) synovial tissues, only minimal programmed cell death of lymphocytes is observed despite intensive infiltration of activated lymphocytes (8). It has also been shown that the resistance of autoreactive B cells to apoptosis in RA is conferred by the up-regulation of Bcl-x\textsubscript{L} expression (9). Collagen-induced arthritis (CIA) is an experimental model of autoimmune disease that shares many of the histologic and immunologic characteristics found in human RA (10, 11). Both cellular and humoral immune responses to type II collagen (CII) are involved in the pathogenesis of CIA. The development of polyarthritis in the CIA model depends on CD4+ T cell activation. T\textsubscript{H}1 cytokine profile (IFN-\gamma, TNF-\alpha, and IL-12) predominates during the induction and acute phase of the disease, while T\textsubscript{H}2 response (IL-4, IL-5, and IL-10) is associated with the remission of the disease (12, 13). Although humoral immunity is important in the development of CIA, the mechanisms by which B cells are involved in the initiation and progression of CIA are not well defined. In addition, the role of Bcl\textsubscript{XL} in CIA has not been studied.

In the current study, we demonstrated that Bcl-x\textsubscript{L} transgenic mice developed accelerated and more severe CIA compared with wild-type (WT) mice. Overexpression of Bcl\textsubscript{XL} induced B cells to produce significantly higher levels of IFN-\gamma compared with WT B cells. Furthermore, Bcl\textsubscript{XL}-overexpressed B cells enhanced T\textsubscript{H}1 and Tc1 differentiation in vitro. Thus, these findings indicate a novel role of Bcl\textsubscript{XL}, an apoptosis regulatory molecule, in regulating B cell function and immune responses by modulating cytokine production and T\textsubscript{H}1 response.

Materials and Methods

Animals

Mice transgenic for Bcl-x\textsubscript{L} under control of the IgH chain intron enhancer (1) and transgene-negative littermates (C57BL/6) of both sexes were housed in autoclaved microisolators; provided with sterile bedding, food, and water; and maintained on a 12 h day/night cycle. Animal experimentation was performed in accordance with protocols approved by the Animal Research Committee of the Baylor College of Medicine.

Induction of CIA and evaluation of arthritis

WT littermates and Bcl\textsubscript{XL} transgenic mice on C57BL/6 background (8-2 wk old) were immunized as previously described (14). In brief, mice were injected intradermally at the base of the tail with 100 \mu g (in 100 \mu l) chicken CII (Sigma-Aldrich) dissolved in 0.01 M acetic acid and emulsified in an equal volume of CFA prepared by grinding 100 mg heat-killed Mycobacterium tuberculosis (H37Ra; Difco Laboratories) in 20 ml IFA (Sigma-Aldrich). Three weeks after primary immunization, mice were given the same injection. Mice were observed for the onset of arthritis, and an arthritis index was derived by grading the severity of each paw from 0 to 3 as described (15). The scoring system was based on the degree of swelling and periarticular erythema. The scores of all four paws were added up to yield the arthritic index.

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*Abbreviations used in this paper: GC, germinal center; RA, rheumatoid arthritis; CIA, collagen-induced arthritis; CII, type II collagen; WT, wild type; DTH, delayed-type hypersensitivity; CGG, chicken y-globulin; LN, lymph nodes.

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Delayed-type hypersensitivity (DTH) induction

Mice were immunized with 50 μg chicken γ-globulin (CGG) in 100 μg RIBI adjuvant (Sigma-Aldrich) subcutaneously at the base of tail. Seven days later, mice received 5 μg CGG in 50 μl PBS in the footpad (contralateral footpad received 50 μl PBS). DTH was estimated 18–36 h later by measuring footpad swelling with a dial caliper. Ag-induced swelling was expressed as footpad thickness increase in 0.1 mm units minus the thickness of the control contralateral paw.

Histology

The procedures for freezing tissues, sectioning, and immunohistochemical staining were previously described (15). In brief, joint tissue samples, spleens, and draining lymph nodes (LN) were frozen in OCT embedding medium. Serial, 6-μm-thick frozen sections were cut in a cryostat microtome, thaw mounted onto poly-l-lysine-coated slides, air-dried, fixed in ice-cold acetone for ten minutes, and stored at −80°C. Sections were stained with H&E and examined for the histological changes of inflammation, pannus formation, cartilage, and bone damage.

Detection of anti-CII Abs by ELISA

CII-specific Abs in mouse sera were determined by ELISA as described (14). In brief, microplates were coated with chicken CII overnight and then blocked with 10% FCS. Samples were added and incubated for 1 h at 37°C and washed. HRP-conjugated goat anti-mouse IgG1, IgG2a, and IgM (Southern Biotechnology) were used as secondary detection reagents. Ab titers were determined as the end points when OD values were <2.5-folds of normal control sera on each plate.

Cytokine assays

Levels of IFN-γ, TNF-α, IL-4, and IL-10 were assayed by ELISA kits (BD Pharmingen) according to the manufacturer’s instructions.

Production of IFN-γ was also determined by FACS analysis. Cultured cells were stimulated with 50 ng/ml PMA (Sigma-Aldrich) and 500 ng/ml ionomycin (Sigma-Aldrich) for 5 h with 10 μg/ml Brefeldin A (Sigma-Aldrich) for the final 4 h. Cells then were washed and stained with FITC- or biotin labeled Abs to CD3, CD4, CD8, and B220 (BD Pharmingen), followed by streptavidin-TC (BD Pharmingen). Cells were then fixed with 4% paraformaldehyde at room temperature for 10 min followed by incubation with 0.5% saponin at room temperature for 10 min. Cells were incubated with PE-labeled anti-IFN-γ Ab (BD Pharmingen) for 30 min and acquired by FACSCalibur (BD Biosciences).

Cell purification and stimulation

Splenic B cells from WT or transgenic mice were purified by MACS. In brief, spleen cells were incubated with biotinylated mAbs specific for CD4, CD8, Thy-1, Mac-1, Gr-1, and Ter-119 (all from BD Pharmingen) and labeled T cells, macrophages, dendritic cells, neutrophils, and erythrocyte cells were removed by incubating with streptavidin-microbeads (Miltenyi Biotec GmbH) by passing through a magnetic column.

Purified B cells (≥95% of purity) were cultured at 3 × 10^6 cells/ml in 24-well plates with various combinations of 10 μg/ml anti-CD40 Ab, 10 ng/ml IL-12, or 40 ng/ml IL-18 for 72 h. For B- and T cells coculture experiments, cells were stimulated with 1 μg/ml anti-CD3 Ab, 10 μg/ml anti-CD40 Ab, 10 ng/ml IL-12, and 40 ng/ml IL-18 for 72 h.

Results

Exacerbated CIA in Bcl-xL transgenic mice

To investigate whether overexpression of Bcl-xL in the B cell compartment affects the development and outcome of CIA, Bcl-xL transgenic mice and WT littermates of C57BL/6 background were immunized with chicken CII and monitored for the development of arthritis over a period of 6 mo. Remarkably, transgenic expression of Bcl-xL in the B cell compartment led to an accelerated onset of the disease (Fig. 1A). Even before secondary CIA challenge, a significant number of the mutant mice showed clinical signs of arthritis. Fifteen days after primary immunization, 40% of Bcl-xL transgenic mice had already developed various degrees of arthritis, while no clinical signs of arthritis were observed in control littermates. Five days after secondary immunization, all of the transgenic mice developed arthritis, compared with a 50% incidence in control mice. In addition, the disease persisted much longer in Bcl-xL transgenic mice than in WT control mice (Fig. 1A). At day 150 after immunization, the inflammatory response subsided in all wild type mice but arthritis still remained in all the transgenic mice (Fig. 1A). Thus, in Bcl-xL transgenic mice, arthritis not only peaked faster, but also lasted much longer.

Additionally, Bcl-xL transgenic mice had consistently more severe arthritis compared with WT mice (Fig. 1B). In the induction phase (days 15–32), the clinical scores of arthritis developed in transgenic and control mice were 4.0 ± 1.5 (mean ± SE) and 1.7 ± 0.9, respectively. During the peak of the disease (days 35–78), the average clinical score observed in transgenic mice was 10.6 ± 0.4, vs 7.9 ± 0.9 in littermate controls. In the remission phase (days 88–148), the disease was much more persistent in Bcl-xL transgenic mice with an average severity of 7.6 ± 0.7. In contrast, arthritis scores decreased steadily in WT animals (1.7 ± 0.8, Fig. 1B).

Consistently, histopathologic studies showed that at 13 wk after immunization, the gross morphology of joint tissues in WT littermates had largely recovered (Fig. 1C). The bones and synovial membrane of transgene-negative animals at this stage of disease looked relatively normal with only small numbers of infiltrating cells remaining in the joints. In contrast, in Bcl-xL transgenic mice, severe arthritis persisted (Fig. 1D). Bone destruction, synovial hyperplasia, and large numbers of infiltrating cells were still observed in Bcl-xL transgenic animals. Therefore, overexpression of Bcl-xL in B cells had profound impact on the disease course and disease severity.
Increased severity of CIA in Bcl-x<sub>L</sub> transgenic mice is associated with an increased T cell response and enhanced Th1 cytokine production

To identify the mechanisms responsible for the elevated arthriticogenic response in Bcl-x<sub>L</sub> transgenic mice, we first determined the levels of serum anti-CII Abs in both Bcl-x<sub>L</sub> transgenic and transgene-negative animals. Surprisingly, there was no significant difference in CII-specific Ab levels between transgenic mice and their WT littermates; both groups produced comparable levels of CII-specific IgM, IgG1, and IgG2a Abs (Fig. 2A).

Because CIA is a T<sub>H</sub>1-mediated inflammatory disease, we further analyzed T cell activation and cytokine profile. The draining LN cells were isolated from both groups of CIA mice and restimulated in vitro with CII for 3 days. The results showed that there was a significant increase in cellular proliferation of draining LN cell cultures from Bcl-x<sub>L</sub> transgenic animals as compared with transgene-negative littermates immunized with CII (Fig. 2B). Because the T/B cell ratios in LNs from transgenic and WT mice remained unchanged and Bcl-x<sub>L</sub> overexpression does not confer an advantage in B cell proliferation (16), these results demonstrate that Bcl-x<sub>L</sub> over-expression in the B cell compartment significantly influenced Ag-specific lymphocyte proliferation. This phenomenon is not restricted to CII Ag. When we immunized mice with CGG and analyzed the proliferation of draining LN cells 1 wk later, we found that the LN cells from Bcl-x<sub>L</sub> transgenic mice also exhibited enhanced proliferation upon in vitro restimulation (data not shown).

We further determined the cytokine profile of the draining LN cells from CIA mice. Remarkably, LN cells from Bcl-x<sub>L</sub> transgenic animals with CIA produced significantly higher levels of IFN-γ than cells from nontransgenic CIA animals after in vitro restimulation with CII (Fig. 3A). In addition, cells from transgenic animals also produced higher levels of TNF-α (Fig. 3B), which is considered critical for arthritogenic responses. The production of Th2 cytokines (IL-4 and IL-10) was lower in cultures from Bcl-x<sub>L</sub> transgenic mice than in control littermates (Fig. 3C and D), but the overall levels of IL-4 and IL-10 were low, consistent with the notion that Th2 cytokine production is generally suppressed in CIA.

The results above demonstrated that Bcl-x<sub>L</sub> transgenic mice mounted an enhanced Th1 response. This finding was further supported by DTH assay, which is generally considered a form of cellular immunity induced by T<sub>H</sub>1 cells (17). As described in Materials and Methods, CGG-immunized mice were rechallenged with CGG one week later. The DTH response observed in the footpads of Bcl-x<sub>L</sub> transgenic mice was significantly higher than that in control littermates (Fig. 4). Thus, results from both in vitro and in vivo experiments demonstrate that constitutive expression of Bcl-x<sub>L</sub> in B cells enhances type 1 cytokine production in response to antigenic challenge, which may be one mechanism for the exacerbation of CIA in Bcl-x<sub>L</sub> transgenic mice.

Elevated IFN-γ production by Bcl-x<sub>L</sub> transgenic B cells

To explore the mechanism responsible for an enhanced Th1 response in Bcl-x<sub>L</sub> transgenic mice, we examined the capability of Bcl-x<sub>L</sub> transgenic B cells to produce IFN-γ under different stimuli. The results showed that purified WT B cells produced significant amounts of IFN-γ only in the presence of a combination of anti-CD40, IL-12, and IL-18 (Fig. 5). When cultured with anti-CD40 antibody.
and IL-18 in the absence of IL-12, transgenic B cells, but not WT B cells, produced high levels of IFN-γ (Fig. 5). In the presence of anti-CD40, IL-12, and IL-18, transgenic B cells produced 20 times more IFN-γ than WT B cells. This increased production of IFN-γ in the cultures of Bcl-xL transgenic B cells was not due to increased numbers of transgenic B cells because there was no difference between the numbers of viable cells in cultures with Bcl-xL transgenic and WT B cells (data not shown). Thus, these data demonstrate that Bcl-xL transgenic B cells have greater capacity and higher sensitivity in producing IFN-γ after activation.

Enhanced IFN-γ production in Bcl-xL transgenic B cells directly promotes Th1/Tc1 cell differentiation

To determine whether the enhanced IFN-γ production by transgenic B cells exerts an effect on the activation and differentiation of T cells, we cocultured T cells with WT or Bcl-xL transgenic B cells in the presence of anti-CD3, anti-CD40, IL-12, and IL-18 for 3 days and measured their expression of IFN-γ and activation markers. Remarkably, the number of T cells producing IFN-γ increased significantly when cocultured with Bcl-xL transgenic B cells than with WT B cells. The numbers of both CD4 and CD8 T cells that expressed intracellular IFN-γ were 2–3-fold higher in cultures with Bcl-xL transgenic B cells than with WT B cells (Fig. 6), demonstrating that transgenic B cells and/or their products strongly promote Th1 and Tc1 differentiation. To investigate whether transgenic B cells influence the activation status of T cells, we examined the expression of various activation markers on T cells in the same cultures. We found that there were no significant differences in surface expression of activation markers such as CD25, CD62L, CD69, ICOS, and CTLA-4 (data not shown). In addition, when T cells labeled with CFSE were cultured, we found that the proportions of T cells that have undergone cell division were comparable between cultures with transgenic and WT B cells (data not shown).

Thus, our data show that overexpression of Bcl-xL in B cells enhances Th1/Tc1 cell differentiation but has little effect on cell activation or cell cycle progression.

Discussion

A balance between lymphocyte proliferation and programmed cell death is essential for the normal function of the immune system. Genes that regulate apoptosis are critical not only for development and the maintenance of cellular homeostasis, but also in the pathogenesis of many diseases including cancer, autoimmune diseases, and viral infections. It has been demonstrated that apoptosis is limited in the RA synovial tissue and arthritogenic lymphocytes are largely resistant to apoptosis (8). In particular, autoreactive B cells in the inflamed joints gain resistance to apoptosis induction by up-regulating Bcl-xL expression (9). In this study, we demonstrate that overexpression of Bcl-xL in B cells exerts a great effect on T cell differentiation and significantly influences the pathogenesis of CIA. In mice that constitutively express Bcl-xL in the B cell compartment, immunization with CII induced more severe arthritis with an earlier onset and longer duration of the disease compared with nontransgenic littermates, accompanied by enhanced Th1 response.

In the CIA model, immunization with CII generates a spectrum of Abs with similar reactivities as found in human RA (18). It has also been shown that passive transfer with serum from CII-immunized animals or with purified anti-CII Abs can cause arthritis in syngeneic recipients (19–21). Therefore, our initial hypothesis was that the more severe arthritis in transgenic animals was mainly a result of enhanced Ab response to the immunizing CII due to the survival advantage of B cells overexpressing Bcl-xL. Surprisingly, the results showed that Bcl-xL transgenic mice produced similar levels of CII specific IgM, IgG1, and IgG2a Abs. An earlier work has demonstrated that overexpression of Bcl-xL in B cells does not change the magnitude of the GC response or the frequency of Ab-forming cells in the bone marrow (16). In fact, over-expression of Bcl-xL in the B cell compartment actually reduces affinity maturation of serum Abs due to a disrupted process of negative selection against low-affinity B cells (16). Thus, overexpression of Bcl-xL in B cells does not enhance Ab response.

Th1 responses and Th1 cytokines such as IL-12, IFN-γ, and TNF-α play an arthritogenic role in CIA and human RA (22–25). Therefore, a possible mechanism to explain the exacerbated arthritogenic response in Bcl-xL transgenic mice is that overexpression of Bcl-xL in the B cell compartment enhances Th1 response. Indeed, Ag-specific proliferation of LN cells from the transgenic mice with CIA was significantly increased compared with that from WT mice with CIA. Because proliferative responses of Bcl-xL transgenic B cells are not elevated compared...
with WT B cells (16), our current data suggest that the proliferation of T cells from Bcl-xL transgenic mice is significantly increased in response to recall Ags. In addition, Bcl-xL transgenic mice with CIA had elevated levels of Tg4 cytokines in draining LN cultures. The enhanced Tg4 response in Bclxl transgenic mice was further supported by an enhanced DTH response, which is commonly believed to be Tg4 mediated. Taken together, these results suggest that enhanced Tg4 response is one mechanism responsible for the more severe and persistent arthritic response in Bclxl transgenic mice.

Our data raised one critical question. How could overexpression of Bcl-xL in B cells lead to a critical type 1 response? One mechanism by which B cells regulate T cell differentiation into Th1 or Th2 cells is through their production of IFN-γ (26). Therefore, we further investigated the ability of Bcl xl transgenic B cells to produce IFN-γ and to promote Th1 differentiation. Indeed, when cultured with anti-CD40 Ab, IL-12 and IL-18, Bclxl transgenic B cells produced significantly higher levels of IFN-γ. Even in the absence of IL-12, Bcl-xL transgenic B cells were capable of producing significant levels of IFN-γ when stimulated with anti-CD40 and IL-18, a combination that only induced a minimal IFN-γ production in WT B cells. Moreover, coculturing T cells with Bcl-xL transgenic B cells resulted in a markedly enhanced T cell differentiation to the Th1/Thc1 pathway. IFN-γ can promote arthritogenic response by enhancing Th1 cell differentiation, activation of macrophages, Ag presentation, and expression of MHC molecules (27–30). However, IFN-γ has other contradictory immunoregulatory roles in the development and progression of CIA (31, 32). Our current data are consistent with the disease-promoting role of IFN-γ in CIA and suggest that overexpression of Bcl-xl promotes IFN-γ production in B cells, and that there is a direct effect of B cell cytokine production on T cell differentiation and function. However, it is not clear why Bcl-xL overexpression promotes IFN-γ production. The link between Bcl-xL overexpression and IFN-γ production in B cells needs further investigation.

Interestingly, one earlier study has shown that T cell lineage-specific expression of Bcl-xl transgene resulted in decreased IFN-γ production and attenuated CIA (33). Several factors may explain the seemingly contradictory results from these two studies. First, the T cell lineage-specific Bcl-xl transgene was under the control of proximal lck promoter (34), which is active mostly in developing T cells, whereas the Bcl-xl transgene used in the current study was under the control of the Ig intron enhancer promoter/enhancer (1). In addition, it is also conceivable that there are differences in the level of Bcl-xl expression in these two Bcl-xl transgenic mouse lines. Furthermore, the lck promoter-controlled Bcl-xl transgenic mice were on DBA/1 (H-2b) genetic background, whereas the transgenic mice used in this study were on C57BL/6 (H-2b) background. It is possible that constitutive expression of Bcl-xl may have different effects on different genetic backgrounds.

In summary, this study provides evidence that B cells play a pathogenic role in CIA via promoting Tg4 response and inflammatory cytokine production. To our knowledge, this is the first time to demonstrate that Bcl-xl, a molecule important in controlling B cell survival, has a significant role in regulating cytokine production and Tg4 response, but not Ab production. These findings may have important therapeutic implications. In human RA, there is evidence that inflammatory cells in the diseased joints are relatively resistant to apoptosis induction by up-regulation of antiapoptotic genes including Bcl-xl (35). Therapeutic approaches using depleting mAbs against T cells or removing B cell products such as Abs and immune complexes have not been particularly effective in RA (36). Given the fact that activated B cells can function as APCs for self-Ags and can also regulate T cell activation and differentiation by cytokine production, controlled apoptosis induction in autoreactive B cells may constitute an effective therapeutic strategy in autoimmune arthritis.

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**Disclosures**

The authors have no financial conflict of interest.

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