Relaxed Negative Selection in Germinal Centers and Impaired Affinity Maturation in *bcl-x*$_L$ Transgenic Mice

By Yoshimasa Takahashi,* Douglas M. Cerasoli,* Joseph M. Dal Porto,* Michiko Shimoda,* Robert Freund,* Wei Fang,‡ David G. Telander,‡ Erika-Nell Malvey,‡ Daniel L. Mueller,‡ Timothy W. Behrens,‡ and Garnett Kelsoe§

*From the *Department of Microbiology and Immunology, University of Maryland School of Medicine, Baltimore, Maryland 21201; the ‡Center for Immunology, Department of Medicine, University of Minnesota Medical School, Minneapolis, Minnesota 55455; and the §Department of Immunology, Duke University Medical Center, Durham, North Carolina 27710*

Summary

The role of apoptosis in affinity maturation was investigated by determining the affinity of (4-hydroxy-3-nitrophenyl)acetyl (NP)-specific antibody-forming cells (AFCs) and serum antibody in transgenic mice that overexpress a suppressor of apoptosis, Bcl-x_L, in the B cell compartment. Although transgenic animals briefly expressed higher numbers of splenic AFCs after immunization, the *bcl-x*$_L$ transgene did not increase the number or size of germinal centers (GCs), alter the levels of serum antibody, or change the frequency of NP-specific, long-lived AFCs. Nonetheless, the *bcl-x*$_L$ transgene product, in addition to endogenous Bcl-x_L, reduced apoptosis in GC B cells and resulted in the expansion of B lymphocytes bearing VDJ rearrangements that are usually rare in primary anti-NP responses. Long-lived AFCs bearing these noncanonical rearrangements were frequent in the bone marrow and secreted immunoglobulin G_1 antibodies with low affinity for NP. The abundance of noncanonical cells lowered the average affinity of long-lived AFCs and serum antibody, demonstrating that Bcl-x_L and apoptosis influence clonal selection/maintenance for affinity maturation.

Key words: Bcl-x_L • apoptosis • affinity maturation • germinal center • clonal selection

A distinct property of humoral immune responses to T cell–dependent antigens is a progressive increase in antibody affinity known as affinity maturation (1, 2). Affinity maturation is achieved by two key events: the generation of antibody variants by V(D)J hypermutation and the subsequent selection of those variants that bind antigen strongly (3, 4). It is widely believed that the selective accumulation of high-affinity B cells is mediated by inter- and intraclonal competition for antigen retained on follicular dendritic cells (FDCs)[1] in germinal centers (GCs) (5–7). However, little is known about the cellular and molecular mechanisms underlying this selection.

GCs serve as a crucial site for antigen-driven V(D)J hypermutation (8, 9). Clonal selection and affinity maturation within this mutated population can be followed by a variety of methods (10, 11) to show that increased affinity is achieved by both preferential retention of higher-affinity B cells (positive selection) and loss of low-affinity B cells (negative selection). Although positive selection can only result from an active process, i.e., selective proliferation, negative selection can arise passively. For example, limiting amounts of antigen may be insufficient to activate B cells with low-affinity receptors (12) or to support their interaction with T lymphocytes (13). Such clones would be rapidly overgrown in the GC population by higher-affinity competitors. Nonetheless, negatively selected GC B cells are believed to die by apoptosis, because GCs are sites of considerable cell death, and in vitro, GC B cells undergo programmed cell death in the absence of activating stimuli (14). Furthermore, administration of large amounts of soluble antigen sharply elevates the number of apoptotic B cells in GCs, an experiment thought to mimic the negative selection of autoreactive mutants (15–17).

Several molecules that regulate apoptosis have been proposed to modulate negative selection during affinity maturation. Bcl-2, an inhibitor of apoptotic cell death, is selectively downregulated in GC B cells (18), and human GC B cells rapidly become apoptotic in ex vivo culture. How-

[1]*Abbreviations used in this paper:* AFC, antibody-forming cell; BCR, B cell antigen receptor; BM, bone marrow; BrdU, 2-bromodeoxyuridine; CG, chicken γ-globulin; ELISPOT, enzyme-linked immunospot; FDC, follicular dendritic cell; GC, germinal center; HRP, horseradish peroxidase; mIg, membrane Ig; NP, (4-hydroxy-3-nitrophenyl)acetyl; PNA, peanut agglutinin; R/S ratio, ratio of replacement to silent mutations; TUNEL, terminal deoxynucleotidyl transferase–mediated dUTP-biotin nick end labeling.

J. Exp. Med. © The Rockefeller University Press • 0022-1007/99/08/399/11 $5.00
Volume 190, Number 3, August 2, 1999 399–409
http://www.jem.org

ever, stimulation of human GC B cells with antibody to membrane Ig (mIg) or CD40 extends the survival of cultured GC cells and upregulates Bcl-2 (14). Reciprocally, a positive regulator of apoptotic cell death, Fas (CD95), is highly expressed in GC B cells (18, 19), and GC B cells are susceptible to Fas-mediated apoptosis in vitro (20, 21). Despite these in vitro models, studies of genetically modified mice do not support major roles for Bcl-2 or Fas in affinity maturation. Neither the overexpression of Bcl-2 nor the lack of Fas has detectable effects on the affinity maturation of serum antibodies (19, 22). These findings raise the possibility that affinity maturation is achieved solely by positive selection, or that other apoptosis-regulatory molecules are involved in the negative selection process.

A homologue of *bcl-2*, *bcl-x*, also suppresses apoptosis through its Bcl-x_L product (23). Bcl-x_L is highly expressed in pre-B cells but is downregulated when B cells enter the mature pool (24, 25). While Bcl-2 plays a critical role in the survival of mature naive lymphocytes (26), Bcl-x_L is important for the survival of immature lymphocytes (27). Interestingly, cross-linking of mIg or CD40 on splenic B cells upregulates the expression of Bcl-x_L (25, 28). Human GC B cells are also known to reexpress Bcl-x_L, with expression confined to the centrocyte subset in which clonal selection is thought to occur (29, 30). These data and the many shared characteristics of immature and GC B cells (31) suggest that Bcl-x_L might control life-or-death decisions in the GC compartment.

To examine the roles of apoptosis and Bcl-x_L in affinity maturation, we tracked the affinity of antibody-forming cells (AFCs) in the bone marrow (BM) and serum antibody of *bcl-*x_L transgenic mice and their congenic wild-type controls, during the clonally restricted antibody response to the (4-hydroxy-3-nitrophenyl)acetyl (NP) hapten (32). Our study revealed that overexpression of Bcl-x_L did not change the magnitude of the GC response or the frequency of AFCs in the BM. However, apoptotic cell death in GCs was significantly reduced in *bcl-*x_L transgenic mice and led to the persistence of many B cells carrying VDJ rearrangements that are normally rare in the later stages of the primary anti-NP response and generally encode lower-affinity antibody (11, 12, 32). Affinity maturation of serum IgG_1 antibody was reduced in transgenic mice by the persistence of low-affinity BM AFCs. Selective apoptosis, rather than the overgrowth of high-affinity clones, appears to be the prime agency by which low-affinity B cells are lost from humoral responses.

Materials and Methods

Antigens and Antibodies. The succinic anhydride ester of NP was reacted with chicken γ-globulin (CG; Sigma Chemical Co.) or BSA (U.S. Biochemical Corp.) as described (32). The coupling ratio of each conjugate was determined spectrophotometrically. Antibodies specific for IgM^b (AF6-78) and mouse λ1 L chain (Ls136) were purified over protein G–Sepharose (Amersham Pharmacia Biotech) from culture supernatants. Horseradish peroxidase (HRP)-conjugated goat anti–mouse IgG_1 and biotinylated anti-IgD antibodies were purchased from Southern Biotechnology Associates. Anti-FcγRI/RII (2.4G2), FITC-labeled GL-7, PE-conjugated anti-B220, biotinylated anti–Mac-1, –Gr-1, -Thy1.2, -CD4, -CD8, and -Ter119, and PE-conjugated anti-CD138 (syndecan) antibodies were purchased from PharMingen. Anti–Bcl-x and anti–Bcl-2 mAbs were purchased from Transduction Laboratories.

Mice and Immunizations. *bcl-*x_L transgenic mice were generated as described previously (24) and backcrossed with the C57BL/6 strain (The Jackson Laboratory). *Igh*^{b} progeny were used in all experiments. *bcl-*x_L transgenic or transgene-negative littermate control mice were immunized intraperitoneally with 50 μg of an NP_{20}-CG conjugate precipitated in alum.

Enzyme-linked Immunospot Assay and Affinity Estimates. The frequencies of NP-specific AFCs in spleen and BM were estimated by enzyme-linked immunospot (ELISPOT) assay using two different coupling ratios of NP-BSA (11). In brief, splenocytes (10^5 cells/well) or BM cells (5×10^5 cells/well) were incubated on nitrocellulose filters coated with NP_2-BSA, NP_{23}-BSA, or BSA alone at 37°C, 5% CO_2 for 2 h. After washing, filters were stained with HRP-conjugated anti-IgG_1 antibodies, and HRP activities were visualized using 3-aminoethyl carbazole. The frequencies of high-affinity and total AFCs were determined from NP_2-BSA– and NP_{23}-BSA–coated filters after background on BSA-coated filters was subtracted.

ELISA Titration of Serum IgG_1 Antibody and Affinity Estimates. IgG_1 specific for the NP hapten was detected by ELISA using two different coupling ratios of NP-BSA as described (11). In brief, serially diluted sera were added to plates coated with NP_2-BSA or NP_{23}-BSA and incubated at 4°C overnight. After washing, HRP-conjugated goat anti–mouse IgG_1 was added, and HRP activity was visualized using a TMB peroxidase substrate kit (Bio-Rad Laboratories). The concentrations of anti-NP IgG_1 antibodies were estimated by comparison with standard curves created from the H33Lγ1/λ1 control antibody on each plate (12). To estimate the affinity of NP-binding antibody in the sera, ratios of NP_2-binding antibody to NP_{23}-binding antibody were calculated.

Histology. All histological procedures were conducted as described previously (32). The number of $\lambda1^+$ GCs was determined by staining sections with biotinylated Ls136, followed by alkaline phosphatase–conjugated streptavidin (Southern Biotechnology Associates) and HRP-conjugated peanut agglutinin (PNA). Apoptotic cell death in GCs was estimated by terminal deoxynucleotidyl transferase–mediated dUTP-biotin nick end labeling (TUNEL) as described (17). $TUNEL^+$ cells in GCs were counted at 200× magnification by systematic scanning. Cell proliferation in GCs was determined by the incorporation of 2-bromodeoxyuridine (BrdU) as described (33). In brief, 10 d after immunization, *bcl-*x_L transgenic and control mice were given 1.0 mg BrdU by intraperitoneal injection; 2 h later, the mice were killed and their spleens prepared for histology. Proliferation indices were determined by microscopic inspection as the fraction (%) of PNA-binding (PNA^+) cells that exhibited nuclear BrdU incorporation.

Sequence Analysis of VDJ Rearrangements from GC B Cells. $\lambda1^+$ GC cells were microdissected from day 12 spleen sections of *bcl-*x_L transgenic mice and control mice. VDJ DNA was amplified by PCR and cloned into Bluescript plasmid (34). The frequency of VDJ genes using the V186.2 gene segment was estimated by colony hybridization using oligonucleotides specific for V186.2 and one specific for the framework 3 region of the mouse V_H V186.2 and V3 subfamilies of the J558 group (32, 35). Plasmid DNA was extracted from ≤3 bacterial colonies from each GC, and V_H gene sequences were determined by automated sequencing.

Flow Cytometry. Single cell suspensions of splenocytes and BM cells were prepared as described (11). Cells were then washed in PBS (pH 7.4) containing 2% FCS and 0.08% sodium azide at 4°C for cytometric analysis, or washed with deficient RPMI 1640 (Irvine Scientific) containing 2% FCS for sorting. The enumeration of GC B cells and sorting of BM AFCs were carried out as described (11).

To collect GC B cells, splenocytes pooled from four mice were blocked with anti-FcγRI/RII and then stained with biotinylated anti-IgD, –Mac-I, –Gr-1, -Thy1.2, -CD4, -CD8, and -Ter119 antibodies for 30 min. After three washes, cells were incubated with streptavidin-conjugated microbeads (Miltenyi Biotec) for 15 min. Cells attached to microbeads were depleted by passage through a CS column (Miltenyi Biotec) in a magnetic field based on the manufacturer's protocol. Recovered cells were stained with FITC-labeled GL-7, PE-conjugated anti-B220, Tricolor-conjugated streptavidin (Caltag Laboratories), and 7-amino-actinomycin D (7-AAD). Finally, GL-7$^+$B220$^+$ cells within the Tricolor$^-$7-AAD$^-$ fraction were sorted into biotin-deficient RPMI containing 2% FCS using a FACStar$^{Plus™}$ (Becton Dickinson). GL-7$^-$B220$^+$ cells were sorted from the same pooled splenocytes without preenrichment by depletion with magnetic beads. To collect B220$^+$ cells and CD3$^+$ cells, splenocytes from naive *bcl-x*$_L$ transgenic and control mice were blocked and stained with FITC-labeled anti-CD3 and PE-conjugated anti-B220 antibody, and then single positive cells were sorted by the same procedure. Sorting routinely yielded populations of >97% purity.

Western Blots. Cells were lysed in a buffer containing 137 mM NaCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 20 mM Tris-HCl (pH 9.0), 1% NP-40, and 10% glycerol. After homogenization, cells were centrifuged and supernatants were recovered. The cell lysates were loaded (10 μg protein) onto 12% SDS polyacrylamide gel and resolved by electrophoresis. The proteins were then transferred by electrophoresis onto polyvinylidene fluoride membranes. After blocking with TBS (Tris-buffered saline) containing 5% nonfat dry milk, the membranes were incubated with 1:500 diluted anti–Bcl-x or anti–Bcl-2 mAbs, then washed five times with TBS containing 0.1% Tween-20. After incubation with 1:20,000 diluted HRP-conjugated goat anti–mouse Ig antibodies (Amersham Pharmacia Biotech), the reaction was developed by enhanced chemiluminescence using the ECL kit (Amersham Pharmacia Biotech) and detected by exposure to X-ray film.

Results

bcl-x$_L$ Transgene Protects B Cells from Passive Apoptotic Death. Spleens of mice carrying the *bcl-x*$_L$ transgene are ~50% larger and contain 30% more mononuclear cells than those of nontransgenic littermates. This increased cellularity is due to a near doubling in the number of mature IgM$^+$ B220$^+$ cells (Fig. 1 A). Flow cytometric analyses of splenic B cells from transgenic mice revealed that expression of IgM, IgD, CD19, CD21, CD22, CD23, and CD24 was identical to that of control littermates (data not shown). Despite the increased numbers of peripheral B lymphocytes, transgenic animals displayed normal levels of serum IgM (1,242 ± 351 vs. 1,295 ± 379 μg/ml) and IgG (1,341 ± 101 vs. 1,777 ± 379 μg/ml) as measured by specific ELISA. As expected (24), the thymic and peripheral T cell compartments of transgenic mice were normal in size and cellular composition (Fig. 1 A, and data not shown).

Initial assays were performed to assess the ability of transgene-bearing B cells to survive in culture medium containing little FCS. Purified splenic B cells from transgenic mice showed a significant survival advantage over control cells

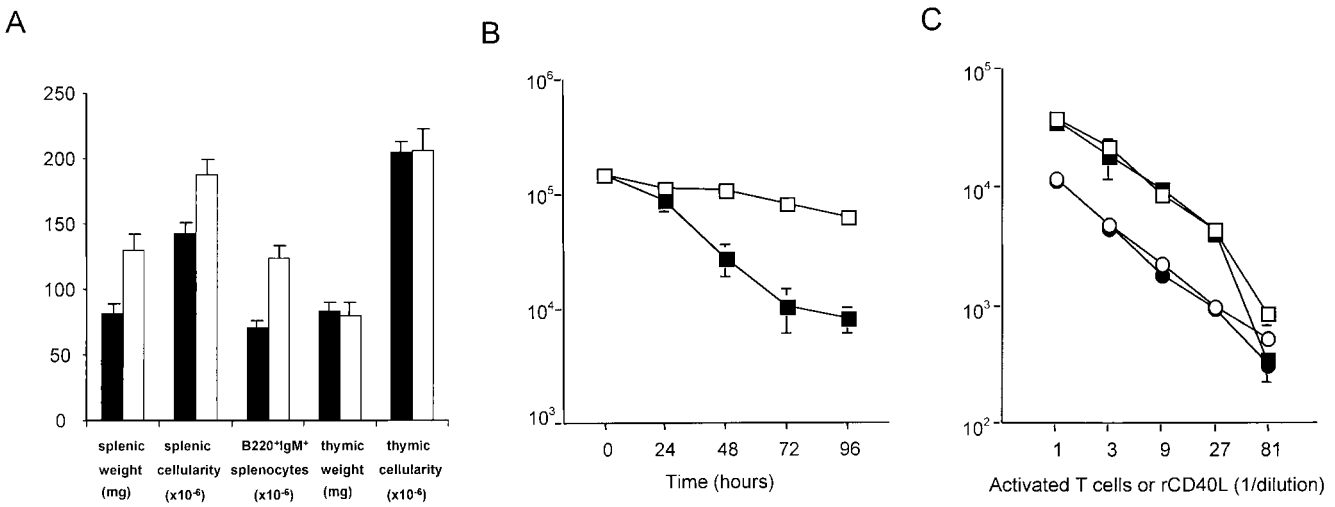

Figure 1. B cells from *bcl-x*$_L$ transgenic mice are resistant to passive cell death and proliferate in vitro in response to activated helper T cell clones or recombinant CD40L. (A) The *bcl-x*$_L$ transgene expands the peripheral B cell compartment. Transgenic animals exhibit increased splenic cellularity that reflects a near doubling of naive IgM$^+$ B lymphocytes. In contrast, thymic weight and cellularity are unchanged. Values represent mean ± SEM of six to nine *bcl-x*$_L$ transgenic mice or wild-type littermate controls. Splenic weight, cellularity, and B cell number are significantly ($P < 0.01$) greater in transgenic mice than in normal controls. (B) Splenic B cells were purified from control (filled symbols) or transgenic (open symbols) mice and cultured in medium containing 1% FCS for 96 h. Viable cells present in triplicate cultures were enumerated by trypan blue exclusion at the indicated times; each point represents the mean number (± SD) of viable B lymphocytes. (C) Purified control and transgenic B cells (1.5×10^5 cells/well) were cultured for 48 h in the presence of helper T cells activated by immobilized CD3-specific antibody (squares) or rCD40L (circles). [^{3}H]Thymidine uptake by the cultured cells was then determined to estimate cellular proliferation. Stimulating T cells were diluted threefold from 3×10^4 cells/well, and medium enriched for rCD40L was serially diluted in threefold steps from 0.3%.

when cultured in medium containing 1% (Fig. 1 B) or 0.1% serum (not shown), indicating their strong resistance to the effects of serum starvation.

Despite their resistance to serum starvation, transgenic B cells displayed no evidence for increased proliferation in response to CD40 cross-linking or T cell help in vitro (Fig. 1 C). In addition, proliferative responses and antibody production in cultures containing LPS were the same for both transgenic and control splenocytes.

Expression Pattern of Endogenous and Transgenic Bcl-x_L in Splenic Lymphocytes. The product of the *bcl-x_L* transgene carries a short epitope tag at its NH_2 terminus and migrates more slowly in SDS-PAGE gels than endogenous Bcl-x_L, which runs as a doublet at ≈31 and 32 kD. Fig. 2 A illustrates that transgenic Bcl-x_L is expressed almost exclusively in the $B220^+$ fraction of splenic lymphocytes. To define transgene expression in B cells participating in an immune response, C57BL/6 mice were immunized with NP-CG, and 12 d later splenic $B220^+$ cells were sorted into $GL\text{-}7^-$ and $GL\text{-}7^+$ fractions to identify follicular and GC B cells, respectively (31). The expression of endogenous Bcl-2 and of endogenous and transgenic Bcl-x_L in these populations were then compared (Fig. 2 B).

Follicular B ($GL\text{-}7^-B220^+$) cells from wild-type mice are positive for Bcl-2 but express little Bcl-x_L. Both proteins are abundant in the follicular population of mice carrying the *bcl-x_L* transgene. In contrast, GC B cells ($GL\text{-}7^+$ $B220^+$) isolated from both wild-type and transgenic mice abundantly express Bcl-x_L but little or no Bcl-2. This observation is consistent with studies of GCs in humans (18, 30). Thus, the reciprocal expression of Bcl-x_L and Bcl-2 observed in pre-B cells holds for GC B cells (24, 25). Interestingly, although transgenic Bcl-x_L is strongly expressed in follicular B cells, only modest amounts of tagged Bcl-x_L could be demonstrated in GC B cells. Reverse transcription PCR studies confirm lower steady state levels of transgenic Bcl-x_L message in the $GL\text{-}7^+$ B cell population (data not shown). This biased expression of transgenic Bcl-x_L may represent distinct Eμ activity in each B cell compartment (the density of mIg on GC B cells is ≤10% of that found on follicular B cells [29]) or downregulation of the transgene's herpes TK promoter in activated cells (24).

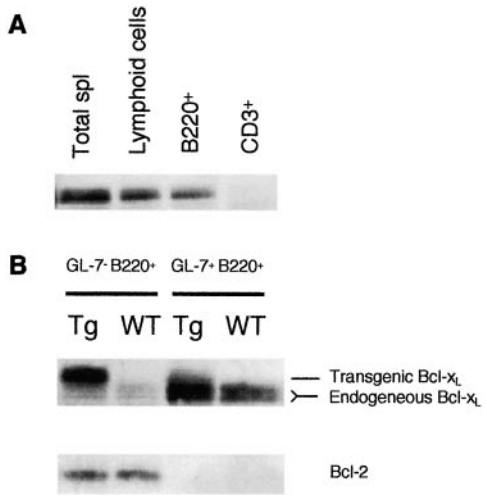

Figure 2. Expression of endogenous and transgenic Bcl-x_L in splenocytes. Expression patterns of Bcl-x_L and Bcl-2 were determined by Western blotting of splenocytes from *bcl-x_L* transgenic mice or wild-type littermate controls. (A) Unselected (total spenocytes [Total spl], lane 1), lymphoid gate (lane 2), and sorted ($B220^+CD3^-$, lane 3; $B220^-CD3^+$, lane 4) splenocytes were recovered from naive transgenic mice. Most, if not all, transgenic Bcl-x_L protein detected in these populations is confined to splenic B lymphocytes. (B) Spleen cells from four immunized transgenic or wild-type mice were pooled at day 12 after immunization. 10 μg of cell lysate protein was immunoblotted using anti–Bcl-x (upper panel) or anti–Bcl-2 (lower panel) mAbs. Molecular masses: transgenic Bcl-x_L, 33 kD; endogenous Bcl-x_L, 31, 32 kD; Bcl-2, 26 kD. Transgenic Bcl-x_L protein is abundant in follicular ($GL\text{-}7^-B220^+$) B cells, but only modest amounts are present in GC B lymphocytes ($GL\text{-}7^+B220^+$). Endogenous Bcl-x_L is expressed at low levels in follicular B cells but is upregulated in the GC. In contrast, Bcl-2 is present in quantity in the follicular population but undetectable in GC B cells.

GCs Develop Normally in bcl-x_L Transgenic Mice. To assess the effects of the *bcl-x_L* transgene on GC development, we compared the GC reaction of transgenic and control mice at day 12 after immunization with NP-CG. This antigen elicits a characteristic hapten-specific antibody that bears a λ1 L chain and an H chain encoded by a canonical VDJ gene rearrangement (32). We identified $\lambda1^+$ GCs in spleen sections by labeling with PNA and anti-λ1 antibody (32) and determined the average number of $\lambda1^+$ GCs per section from groups of transgenic and control mice (Fig. 3 A). Differences in the number or size (data not shown) of GCs were not observed between the groups, nor did the mean frequency of $\lambda1^+$ GCs significantly differ between transgenic (35.4%) and wild-type mice (41.7%). When transgenic mice were immunized with carrier protein alone, the average frequency of $\lambda1^+$ GCs was 7.6%. Thus, frequent $\lambda1^+$ GCs in transgenic mice result from immunization with NP rather than altered λ1 L chain expression.

The frequencies of splenic GC B cells ($GL\text{-}7^+B220^+$ fraction) in transgenic and control mice were also determined by flow cytometry. Both groups supported equivalent and typical GC responses (Fig. 3 B): in transgenics, the frequency of GC B cells peaked at an average of 2.46% of splenocytes compared with 2.39% in controls at day 12 after immunization. Proliferative activity in the GC compartments of both transgenics and controls was also equivalent; 10 d after immunization, 21 vs. 24% of PNA^+ GC cells were labeled by a 2-h pulse of BrdU (not shown).

Transgenic Bcl-x_L Increases Splenic AFC Numbers but not Their Longevity or Serum Antibody Titers. T cell–dependent antigens induce two distinct populations of AFC, a short-lived splenic population that generates the earliest primary antibody and a long-lived set in the BM that maintains the serum response (10, 11). Frequencies of NP-specific, IgG_1 AFCs in the spleen and BM of transgenic and control mice were determined by ELISPOT assay 12, 35, and 69 d after immunization (Fig. 3 C). The kinetics of AFC production were virtually identical in both groups of mice, but splenic AFCs were threefold more abundant in transgenic mice than in controls. This increase may reflect the approximately twofold increase in the number of B cells in the spleens of transgenic mice (Fig. 1 A). Despite their greater

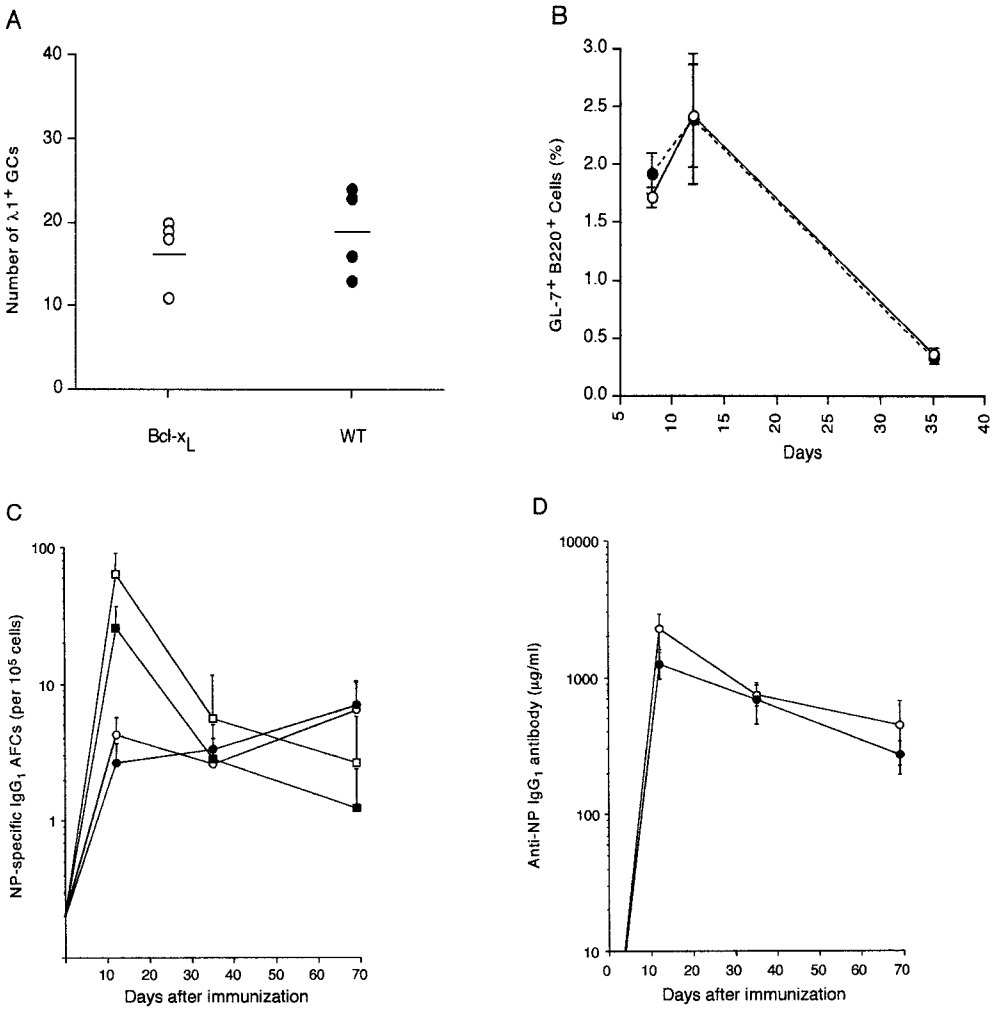

Figure 3. *bcl-x_L* transgenic mice produce GC and IgG_1 antibody responses that are similar to control animals. Splenocytes were recovered from transgenic mice or wild-type controls at various times after immunization with NP-CG. (A) The numbers of $\lambda1^+$ GCs were determined by histological staining with anti-λ1 antibody and PNA. Each point represents the average number of $\lambda1^+$ GCs per histologic section (≥3 sections representing ≈2/3 splenic area) in single transgenic (open circles) or wild-type (filled circles) mice. (B) The percentage of GC B cells in live lymphocytes was assessed by flow cytometry using anti-B220 and anti–GL-7 antibodies. Each point represents the frequency mean (± SD) of GC B cells (percentage of total lymphoid gate) in single transgenic (open circles) and control (filled circles) mice 8, 12, and 35 d after immunization. (C) Numbers of NP-specific AFCs from BM (circles) and spleen (squares) of *bcl-x_L* transgenic (open) or littermate control mice (filled) were determined by ELISPOT using NP_{23}-BSA as the capture antigen. Frequencies of AFCs in naive mice (day 0) from both groups were $<0.2 \times 10^{-5}$. (D) NP-specific serum antibody from transgenic (open) or wild-type control mice (filled) was determined by ELISA using NP_{23}-BSA. The average values (± SD) for serum antibody concentrations from five to seven individual mice per time point are presented.

numbers, splenic AFCs in transgenic mice were lost at the same rate as in control animals. This rapid decline contrasts with *bcl-2* transgenic mice, which support higher numbers and longer-lived splenic AFCs (22). Frequencies and kinetics of specific BM AFCs were indistinguishable between transgenic and control mice (Fig. 3 C).

The expanded splenic AFC pool in transgenic mice resulted in a minor increase in serum antibody titers on day 12, but later levels of antibody did not differ significantly between transgenic and control mice. In both groups, antibody concentrations were at maximal levels on day 12 and then slowly declined to about one third of this peak by day 69 (Fig. 3 D). Thus, overexpression of Bcl-x_L modestly expands recruitment into the splenic AFC pool but does not change cellular recruitment into GCs, entry into the BM AFC pool, or maintenance of long-lasting serum antibody.

bcl-x_L Transgenic Mice Have Fewer Apoptotic Cells in GCs. GCs contain more apoptotic lymphocytes as determined by TUNEL than other regions of spleen (17). These $TUNEL^+$ cells are thought to represent lymphocytes that have been negatively selected during the GC response. We performed TUNEL assays on spleen sections from transgenic and control mice to determine if the small addition of transgenic Bcl-x_L expressed in GC B cells was sufficient to reduce programmed cell death. $TUNEL^+$ cells in GCs from both groups were counted by microscopic examination, and the frequency of $TUNEL^+$ cells per unit area was calculated. These frequencies were subdivided into 12 categories, and the distribution histogram for each category was plotted (Fig. 4). GCs from *bcl-x_L* transgenic mice contained fewer $TUNEL^+$ cells per unit area ($P < 0.01$) than those from control mice (Fig. 4). The most common apoptotic index in wild-type animals was 2.0–2.5 $TUNEL^+$ cells/unit area but only 1.0–1.5 in the *bcl-x_L* transgenics. Perhaps more significantly, >20% of GCs in control mice contained >3 $TUNEL^+$ cells/unit area, whereas only 5% of GCs in *bcl-x_L* transgenic animals held 3.0–4.0 apoptotic cells/unit area with no GCs in the 4.5–6.0 categories. Thus, a modest addition of Bcl-x_L in transgene-bearing GC B cells leads to a readily detectable decrease of $TUNEL^+$ cells.

GC B Cells Using Noncanonical VDJ Rearrangements Are More Frequent in bcl-x_L Transgenic Mice. Initially, immuni-

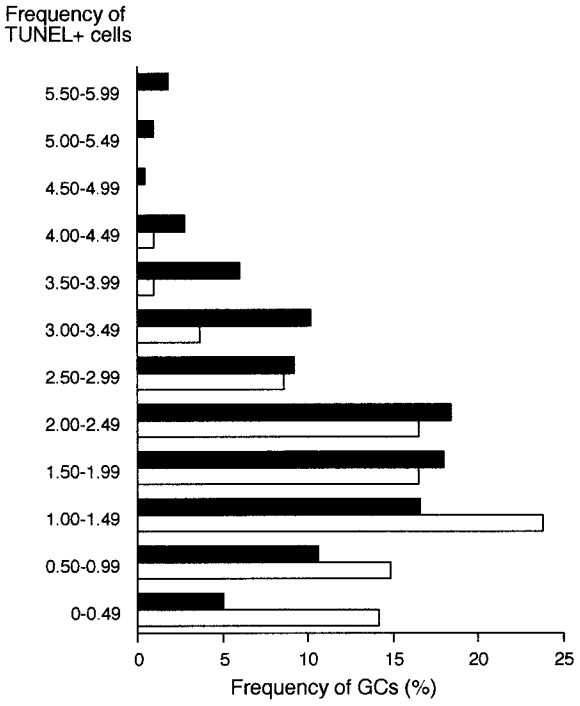

Figure 4. *bcl-x_L* transgenic mice show reduced numbers of TUNEL$^+$ cells in GCs. Spleen sections were prepared from transgenic mice (white bars) and wild-type control mice (black bars) at day 12 after immunization. TUNEL assays were performed with staining by PNA to identify GCs. The number of TUNEL$^+$ cells present and the area of each GC were determined under 200× magnification from >500 GCs. Frequencies of TUNEL$^+$ GC cells/area were then calculated, and each frequency was placed into 1 of 12 categories. The distributions of categories for transgenic and wild-type controls are plotted.

zation with NP conjugates stimulates a broad population of splenic B cells that bear the λ1 L chain and H chain genes made from the V186.2 and V3 subgroups of the J558 V_H gene family (34). Until day 6–7 of the primary response, many GC B cells express V_H gene segments that closely resemble the canonical V186.2 element but encode lower-affinity NP-binding antibodies (12, 34, 36, 37; and Shimoda, M., and G. Kelsoe, unpublished data). By day 10, the majority of B cells bearing these noncanonical V_H gene rearrangements are replaced by higher-affinity cells bearing V186.2/DFL16.1 rearrangements and a tyrosine-rich junctional motif, YYGS (12, 34). Thus, after day 10 the efficiency of affinity-based competition is estimated by the ratio of GC B cells bearing V186.2 versus noncanonical V_H rearrangements. Cells were microdissected from a total of 27 λ1$^+$ GCs from 4 transgenic and 4 control mice 12 d after immunization with NP-CG. VDJ rearrangements from both groups of mice were amplified by PCR and cloned into bacteria, and >600 bacterial colonies were then subjected to hybridization using V186.2-specific and subgroup-specific primers. About 80% (64/81) of VDJ rearrangements recovered from control mice selectively hybridized to the V186.2 V_H gene segment, consistent with previous studies (12, 34), whereas fewer than half (261/554) of the VDJ fragments amplified from *bcl-x_L* transgenic mice exhibited preference for V186.2.

These hybridization data were confirmed by sequencing VDJ inserts from representative bacterial colonies (Table I). From normal C57BL/6 control mice, 79% (11/14) of sequenced VDJ rearrangements contained the V186.2 gene segment, confirming our hybridization analysis and prior sequence studies (33). In contrast, only 47% (17/36) of sequenced VDJ fragments from *bcl-x_L* transgenics carried the canonical V186.2 element (Table I). Noncanonical rearrangements from both control and transgenic mice contained other V_H genes from the V186.2 and V3 subfamilies (35) commonly recovered in early primary anti-NP responses (12, 34).

The use of noncanonical VDJ rearrangements by immunized transgenic mice was not due to altered usage of VH gene segments in naive λ1$^+$ B cells. We recovered splenic λ1$^+$B220$^+$ cells from unimmunized, transgenic, and wild-type mice by fluorescence-activated cell sorting, amplified their VDJ rearrangements with the PCR primers used to study GC populations, and determined the ratios of VDJ rearrangements containing V186.2 versus related V_H genes. There was no significant difference in the percentage of V186.2 genes used by naive Bcl-x_L transgenic mice (14%; 12/88) and naive controls (16%; 12/74). These observations suggest that even slight overexpression of Bcl-x_L in GC B cells leads to lower numbers of TUNEL$^+$ GC cells and the persistence of clones bearing noncanonical VDJ rearrangements that commonly encode low-affinity antibodies present early in the primary response to NP.

The abundance of noncanonical VDJ rearrangements was not associated with impaired positive selection. Patterns of mutation in VDJ rearrangements containing the

Table I. *Somatic Genetics of λ1$^+$ GC Cells in bcl-x_L Transgenic and Wild-type Mice 12 d after Immunization*

	bcl-x_L	Wild-type
V186.2 (% of total)	17 (47%)	11 (79%)
Other (% of total)	19 (53%)	3 (21%)
Average no. of mutations in V186.2	3.5	4.3
R/S ratio		
CDR1 (14.0/1)*	8.0/1	>1.0/1
CDR2 (4.3/1)	>15.0/1	4.8/1
FW (3.1/1)	1.3/1	2.4/1
DFL16.1 (%)‡	59	64
YYGS (%)§	24	18

All mice were immunized with NP-CG. Complete sequence data are available from EMBL/GenBank/DDBJ under accession nos. AF065315-31 (*bcl-x_L*) and AF065332-42 (wild-type).
*R/S ratio of V_H V186.2 given random mutagenesis.
‡Percentage of rearrangements using DLF16.1 gene segments in all rearrangements of V_H V186.2.
§Percentage of rearrangements encoding YYGS in CDR3 in all rearrangements of V_H V186.2.

canonical V186.2 gene segment were similar in transgenic and control mice, with no significant difference ($P > 0.05$) in the ratios of replacement versus silent mutations (R/S ratios) in CDRs (Table I). Other characteristics indicative of high-affinity, NP-specific B cells, e.g., the fraction of rearrangements containing DFL16.1 and the YYGS CDR3 motif, were also similar in both groups. Thus, cellular recruitment, V(D)J hypermutation, and positive selection in GCs are unaffected by the *bcl-*x_L transgene.

Transgenic Bcl-x_L *Leads to Impaired Affinity Maturation in the BM AFC Compartment and Serum Antibody*. Serum antibody is maintained by long-lived BM AFCs that depend on the GC differentiation pathway (10, 11, 38, 39). Affinity maturation of serum antibody and the BM AFC compartment can be monitored by differential binding to ELISA or ELISPOT substrates with sparse (NP_2) or dense (NP_{23}) hapten coatings (10, 11). High-affinity antibody from serum and AFCs binds equally well to both hapten densities, whereas low-affinity binding is evident only on the NP_{23} substrate.

The high-affinity compartment of BM AFCs in wild-type mice rapidly increased between days 12 (30.3%) and 35 (75.6%) of the response, with a more gradual increase up to day 69 (88.4%) (Fig. 5 A). This kinetic is typical of normal responses (11). At day 12, high-affinity AFCs were as common in the BM of transgenic mice (34.3%) as in controls. However, this population expanded more slowly in animals with the *bcl-*x_L transgene, reaching only 57.5 and 60.6% by days 35 and 69, respectively (Fig. 5 A). Remarkably, at day 69 of the response three transgenic mice had smaller high-affinity AFC compartments than were present at day 35, indicating little or no proliferation/survival advantage for high-affinity cells even when antigen concentrations should be minimal.

The average affinity of NP-specific serum antibody was determined for the same mice by ELISA (Fig. 5 B). In wild-type controls, early (day 12) serum antibody contained little or no high-affinity component; by day 35 roughly half of the serum antibody displayed high-affinity binding, and by day 69 this value increased to >90% (Fig. 5 B). The average affinity of serum antibody in transgenic mice also increased from day 12 to day 69, but again the extent of affinity maturation was only ~60% of controls. Overexpression of Bcl-x_L led to diminished affinity maturation in both BM AFCs and the serum antibody.

Impaired Affinity Maturation in Transgenic Mice Is Reflected in the Somatic Genetics of the BM AFCs. To determine the cause of decreased affinity in the BM AFCs of transgenic mice, we recovered the $\lambda 1^+$ BM AFC populations from immunized wild-type ($n = 5$) and transgenic ($n = 5$) mice by cell sorting (11). Typically, at day 69 after immunization >50% of sorted cells from both groups of mice secreted IgG_1 antibody specific for NP. Enriched BM AFC populations were subjected to a reverse transcription PCR that preferentially amplifies cDNA representing rearrangements of the V186.2 and V3 subfamilies of V_H gene segments joined to Cγ1 (11). Amplified VDJ rearrangements were cloned and sequenced to identify the V_H and D gene segments used and any mutations present. Table II summarizes this work and shows that only half (11/21) of the VDJ sequences recovered from *bcl-*x_L transgenic mice used the V186.2 gene segment. In contrast, nearly all (16/17) VDJ rearrangements from wild-type mice contained the V186.2 gene segment. Thus, the high frequency of B cells bearing noncanonical VDJ rearrangements present in day 12 GCs (47%; Table I) was maintained in the day 69 BM AFC population (53%) of mice with the *bcl-*x_L transgene. The reduced average affinity of BM AFCs in *bcl-*x_L transgenic mice results from the retention of B cells bearing noncanonical VDJ rearrangements. Interclonal competition in both

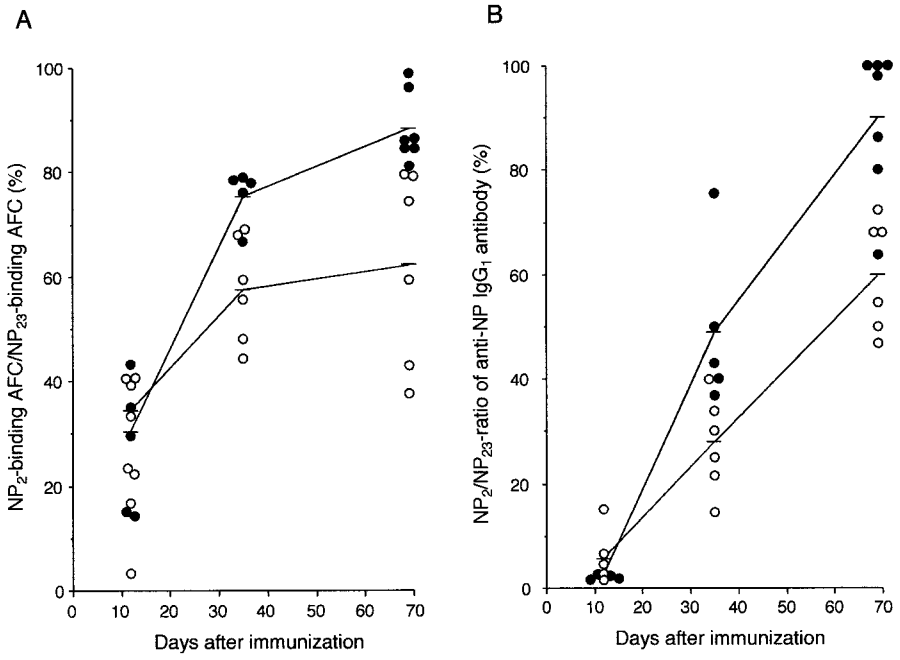

Figure 5. *bcl-*x_L transgenic mice show relaxed affinity maturation of NP-specific IgG_1 BM AFCs and serum antibody. The average affinities of BM AFCs (A) and serum antibodies (B) produced by transgenic (open circles) and control (filled circles) mice at different time points were estimated. (A) The frequencies of NP_2- and NP_{23}-specific IgG_1 AFCs from BM were determined by ELISPOT. Ratios of NP_2 versus NP_{23}-specific AFCs were then calculated and plotted. AFC affinities are significantly ($P < 0.05$) lower in transgenic animals at 35 and 69 d after immunization. (B) Concentrations of NP_2- and NP_{23}-specific IgG_1 antibody were determined by ELISA, and the ratios of NP_2- versus NP_{23}-specific IgG_1 antibody were plotted. Each point represents the ratio determined in a single mouse. The average affinity of serum antibody in transgenic mice is significantly ($P < 0.05$) lower than that of controls at 69 d after immunization.

Table II. *Somatic Genetics of BM AFCs in bcl-x_L Transgenic and Wild-type Mice 69 d after Immunization*

	bcl-x_L	Wild-type
V186.2 (% of total)	11 (52%)	16 (94%)
Other (% of total)	10 (48%)	1 (6%)
Average no. of mutations in V186.2	10.3	4.3
R/S ratio		
CDR1 (14.0/1)*	1.8/1	14.0/1
CDR2 (4.3/1)	3.1/1	8.2/1
FW (3.1/1)	2.1/1	1.5/1
DFL16.1 (% of total)‡	46	69
YYGS (% of total)§	27	44
W→L33 (% of total)‖	18	25

All mice were immunized with NP-CG. Complete sequence data are available from EMBL/GenBank/DDBJ under accession nos. AF065343-63 (*bcl-*x_L) and AF065364-80 (wild-type).
*R/S ratio of V_H V186.2 given random mutagenesis.
‡Percentage of rearrangements using DLF16.1 gene segments in all rearrangements of V_H V186.2.
§Percentage of rearrangements encoding YYGS in CDR3 in all rearrangements of V_H V186.2.
‖Percentage of all V_H V186.2 rearrangements bearing a W→L mutation in codon 33.

the GC and BM AFC compartments of transgenic mice is relaxed, even when the amounts of residual antigen are thought to be limiting.

Comparison of the frequency and pattern of mutations in rearranged V186.2 gene segments from transgenic and control mice suggests that intraclonal selection may also be weakened by increased Bcl-x_L expression. The average frequency of mutations in V186.2 V_H gene segments from the BM AFCs of *bcl-*x_L transgenic mice was about twice as high as that from control mice (Table II). B cells in transgenic mice might better survive the GC environment than B cells in control animals, allowing them to accumulate more mutations before entering the long-lived AFC compartment (11). Furthermore, R/S ratios in both CDR1 and CDR2 of these genes were lower in transgenic mice than in controls. Higher R/S ratios in CDRs arise in part as a consequence of antigenic selection. Other characteristics indicative of high-affinity, NP-specific B cells, such as the percentage of genes bearing DFL16.1, the YYGS CDR3 motif, and a Trp→Leu (W→L) mutation at position 33, were also less common in transgenic mice than in controls. Together, these data show that the *bcl-*x_L transgene enhances the survival of low-affinity GC B cells bearing noncanonical VDJ rearrangements, increases their accumulation of VDJ mutations, and allows low-affinity GC cells to remain in the BM AFC compartment. Persistence of this low-affinity component does not come at the expense of positive selection for higher-affinity clones but by diminished apoptosis in low-affinity B cells.

After day 35, the population of high-affinity BM AFCs grew twice as fast in wild-type mice as in transgenics (Fig. 5) even though both groups supported equivalent numbers of NP-specific, IgG_1 AFCs (Fig. 3). Administration of anti-CD154 antibody to abrogate the GC reaction and reduce the genetic diversity present in the BM AFC compartment (11, 40) enhanced this difference. Control mice that received the CD154-specific antibody, MR1, on days 6, 8, and 10 after immunization had a high-affinity BM AFC compartment of 15.1% at day 22 of the response that grew to 55.8% by day 69 (data not shown). Immunized transgenic mice that received MR1 antibody began with a comparable high-affinity AFC compartment, 12.0% at day 22, but this population grew to only 22.0% by day 69. Thus, overexpression of Bcl-x_L also retards affinity maturation outside the GC microenvironment.

Discussion

In this report, we have demonstrated that a *bcl-*x_L transgene reduces apoptosis in the GC reaction and impairs affinity maturation by sparing cells normally lost from the primary response. This transgene also enhances the survival of peripheral B cells in response to serum starvation in vitro and rescues developing B lymphocytes with aberrant VDJ rearrangements. These effects represent supplementation of endogenous Bcl-x_L activity; although Bcl-x_L is abundant in the GL-7^+B220^+ GC cells of wild-type mice, Bcl-2 is not (Fig. 2 B). Similar observations have been reported for human GC cells where Bcl-x_L rather than Bcl-2 mediates the CD40-dependent survival of centrocytes ex vivo (30).

This result contrasts with that of Bcl-2 overexpression, which does not interfere with affinity maturation (22) but permits the survival of mature autoreactive B cells in the periphery (41). The *bcl-x* and *bcl-2* transgenes also act differently during negative selection in immature B cells, as transgenic Bcl-x_L has the ability to block negative selection and promote developmental maturation, whereas autoreactive cells transgenic for *bcl-2* remain arrested in development (42, 43). Given the similar reciprocal expression of *bcl-2* and *bcl-x* in GC B cells and pre-B cells, *bcl-x* may have a distinct role in regulating the survival of B cells undergoing selection via mIg or the pre-B cell antigen receptor (BCR) (24, 30). Bcl-x_L becomes abundant in B cells after cross-linking mIg or CD40 (25, 28), and the fate of GC B cells is controlled by these same signals (40). We speculate that the degree or quality of mIg signaling in low-affinity B cells does not induce Bcl-x_L expression as effectively as in high-affinity cells, and that this deficit leads to apoptosis. That even a slight addition of transgenic Bcl-x_L to the higher levels of the endogenous protein in GC B cells leads to significant effects on cell death and affinity maturation indicates that GC B cells are quite sensitive to small changes in levels of this death antagonist. The fate of lower-affinity GC B cells appears to be determined by a regulatory threshold of Bcl-x_L.

Relaxed negative selection and the retention of low-affinity B cells in transgenic mice did not alter the duration

or magnitude of the GC response in *bcl-x*$_L$ transgenic mice (Fig. 3). At 35 d after immunization, the splenic GC reaction had ended both in transgenic (0.33% GL-7$^+$B220$^+$ spleen cells) and control (0.37%) animals. This is the earliest time after immunization that the numbers of splenic GL-7$^+$ B220$^+$ cells return to preimmune levels in normal mice (11). Thus, the GC response appears to be regulated by factors beyond affinity-driven competition and selective apoptosis. The rise and fall of GCs depend on the presence of antigen, sustained cell–cell interactions, and cues for cellular location (40, 44–47). It is not surprising that this important immunological response is controlled by finer means than that afforded by Darwinian competition alone.

Nie et al. (37) have reported that immunization of C57BL/6 mice with complexes of antibody and antigen elicits lower-affinity serum antibody and a genetically diverse GC reaction similar to that we observe in *bcl-x*$_L$ transgenic mice. These authors hypothesize that immune complexes decorated with C3d efficiently recruit the CD21/CD19/CD81 coreceptor to antigen-binding BCRs to reduce the threshold of B cell activation. Lowered activation thresholds would result in reduced levels of affinity-driven selection. Although Nie and colleagues describe a phenotype similar to that of *bcl-x*$_L$ transgenic mice, we think it is unlikely that Bcl-x$_L$ reduces selection intensity by enhancing BCR signals. Background levels of IgM and IgG are similar in the serum of transgenic and control mice, and both contain only trace amounts of NP-binding IgG before immunization. Thus, immune complexes are no more likely to form in *bcl-x*$_L$ transgenics than in wild-type controls after primary immunization. Our data do support the notion that enhanced BCR signals, perhaps mediated by coreceptor recruitment, result in reduced apoptosis; immunization with immune complexes may facilitate Bcl-x$_L$ expression in responding B lymphocytes.

A possible mechanism for continued selection by apoptosis outside of GCs is competition among memory B cells for restimulation (48, 49). However, memory B cells are thought to regain Bcl-2 expression, and it would be surprising if their survival depended also on Bcl-x$_L$ (18). Alternatively, selective competition among BM AFCs for antigen might drive sustained affinity maturation. BM AFCs express low levels of mIg (11) and could interact with antigen depots in a Bcl-x$_L$–dependent fashion. Indeed, plasmacytomas express Bcl-x$_L$ (50), and human plasma cells exhibit high levels of Bcl-x$_L$ but low levels of Bcl-2 (51), although it is unclear if these cells represent long- or short-lived AFCs. It will be important to learn how BM AFCs integrate the usually antagonistic processes of differentiation to antibody secretion and cellular longevity so as to maintain protective levels of serum antibody over long time periods.

Our data provide strong evidence of a continuing role for antigen in the maintenance of the long-lived AFC pool. However, Manz et al. (38) have reported that the transfer of BM AFCs into unimmunized recipients reconstitutes long-term serum antibody and conclude that antigen is unnecessary for the survival of these cells. Such experiments are complicated by the possibility of coincidental transfer of residual antigen (6, 7, 48), but we cannot exclude the possibility that post-GC selection acts on precursors of the long-lived AFC pool. In this case, the characteristic somatic genetic changes observed in BM AFCs (11; Tables I and II) would first occur in the precursor population. Such selection would be antigen dependent and affinity driven. Recent work on the longevity and affinity of BM AFCs and serum antibody (our unpublished studies) support the importance of antigen retention and/or BCR signaling in shaping the long-lived AFC population. What remains unchallenged is that affinity maturation of serum antibody continues for months after primary immunization (1, 2, 11; Fig. 5). Although this progressive increase in affinity could be programmed in the early phase of the response, we suggest that in some way antigen continues to exert selection on the responding B cells.

We gratefully acknowledge the assistance of M. Gendelman, and the additional flow cytometric and BrdU-labeling data provided by Dr. B. Zheng. We thank T.F. Tedder, S. Foster, and M. Davila for help with the manuscript.

This work was supported in part by U.S. Public Health Service grants AI24335, AG10207, AG13789 (to G. Kelsoe), CA63111 (to R. Freund), and AR43805 (to T.W. Behrens).

Address correspondence to Garnett Kelsoe, Department of Immunology, Box 3010, Duke University Medical Center, Durham, NC 27710. Phone: 919-613-7936; Fax: 919-613-7878; E-mail: ghkelsoe@duke.edu

Submitted: 16 March 1999 Revised: 1 June 1999 Accepted: 2 June 1999

References

1. Eisen, H.N., and G.W. Siskind. 1964. Variations in affinities of antibodies during the immune response. *Biochemistry*. 3:996–1008.
2. Siskind, G.W., and B. Benacerraf. 1969. Cell selection by antigen in the immune response. *Adv. Immunol*. 10:1–50.
3. Griffiths, G.M., C. Berek, M. Kaartinen, and C. Milstein. 1984. Somatic mutation and the maturation of the immune response. *Nature*. 312:271–275.
4. French, D.L., R.Laskov, and M.D. Scharff. 1989. The role of somatic hypermutation in the generation of antibody diver-

sity. *Science*. 244:1152–1157.
5. Nossal, G.J.V., G.L. Ada, and C.M. Austin. 1964. Antigens in immunity. IV. Cellular localization of ^{125}I and ^{131}I labeled flagella in lymph nodes. *Aust. J. Exp. Biol. Med. Sci*. 42:311–330.
6. Tew, J.G., and T.E. Mandel. 1979. Prolonged antigen half-life in the lymphoid follicles of specifically immunized mice. *Immunology*. 37:69–76.
7. Mandel, T.E., T.E.R. Phipps, A. Abbot, and J. Tew. 1980. The follicular dendritic cell: long term antigen retention during immunity. *Immunol. Rev*. 53:29–59.
8. Jacob, J., G. Kelsoe, K. Rajewsky, and U. Weiss. 1991. Intraclonal generation of antibody mutants in germinal centers. *Nature*. 354:389–392.
9. Berek, C., A. Berger, and M. Apel. 1991. Maturation of the immune response in germinal centers. *Cell*. 67:1121–1129.
10. Smith, K.G.C., A. Light, G.J.V. Nossal, and D.M. Tarlinton. 1997. The extent of affinity maturation differs between the memory and antibody-forming cell compartments in the primary immune response. *EMBO (Eur. Mol. Biol. Organ.) J*. 16:2996–3006.
11. Takahashi, Y., P.R. Dutta, D.M. Cerasoli, and G. Kelsoe. 1998. In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. V. Affinity maturation develops in two stages of clonal selection. *J. Exp. Med*. 187:885–895.
12. Dal Porto, J., A. Haberman, M. Shlomchik, and G. Kelsoe. 1998. Antigen drives very low affinity B cells to become plasmacytes and enter germinal centers. *J. Immunol.* 161:5373–5381.
13. Batista, F.D., and M.S. Neuberger. 1998. Affinity dependence of the B cell response to antigen: a threshold, a ceiling, and the importance of off-rate. *Immunity*. 8:751–759.
14. Liu, Y.J., D.E. Joshua, G.T. Williams, C.A. Smith, J. Gordon, and I.C.M. MacLennan. 1989. Mechanism of antigen-driven selection in germinal centres. *Nature*. 342:929–931.
15. Pulendran, B., G. Kannourakis, S. Nouri, K.G.C. Smith, and G.J.V. Nossal. 1995. Soluble antigen can cause enhanced apoptosis of germinal-centre B cells. *Nature*. 375:331–334.
16. Shokat, K.M., and C.C. Goodnow. 1995. Antigen-induced B-cell death and elimination during germinal-centre immune responses. *Nature*. 375:334–338.
17. Han, S., B. Zheng, J. Dal Porto, and G. Kelsoe. 1995. In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. IV. Affinity-dependent, antigen-driven B cell apoptosis in germinal centers as a mechanism for maintaining self-tolerance. *J. Exp. Med*. 182:1635–1644.
18. Martinez-Valdez, H., C. Guret, O. de Bouteiller, I. Fugier, J. Banchereau, and Y.-J. Liu. 1996. Human germinal center B cells express the apoptosis-inducing genes Fas, c-myc, P^{53}, and Bax but not the survival gene bcl-2. *J. Exp. Med*. 183: 971–977.
19. Smith, K.G.C., G.J.V. Nossal, and D.M. Tarlinton. 1995. FAS is highly expressed in the germinal center but is not required for regulation of the B-cell response to antigen. *Proc. Natl. Acad. Sci. USA*. 92:11628–11632.
20. Liu, Y.-J., C. Barthelemy, O. de Bouteiller, C. Arpin, I. Durand, and J. Banchereau. 1995. Memory B cells from human tonsils colonize mucosal epithelium and directly present antigen to T cells by rapid up-regulation of B7-1 and B7-2. *Immunity*. 2:239–248.
21. Choe, J., H.-S. Kim, X. Zhang, R.J. Armitage, and Y.S. Choi. 1996. Cellular and molecular factors that regulate the differentiation and apoptosis of germinal center B cells. *J. Immunol*. 157:1006–1016.
22. Smith, K.G.C., U. Weiss, K. Rajewsky, G.J.V. Nossal, and D.M. Tarlinton. 1994. Bcl-2 increases memory B cell recruitment but does not perturb selection in germinal centers. *Immunity*. 1:803–813.
23. Boise, L.H., M. Gonzalez-Garcia, C.E. Postema, L. Ding, T. Lindsten, L.A. Turka, X. Mao, G. Nunez, and C.B. Thompson. 1993. *bcl-x*, a *bcl-2*-related gene that functions as a dominant regulator of apoptotic cell death. *Cell*. 74:597–608.
24. Fang, W., D.L. Mueller, C.A. Pennell, J.J. Rivard, Y.S. Li, R.R. Hardy, M.S. Schlissel, and T.W. Behrens. 1996. Frequent aberrant immunoglobulin gene rearrangements in pro-B cells revealed by a bcl-x_L transgene. *Immunity*. 4:291–299.
25. Grillot, D.A.M., R. Merino, J.C. Pena, W.C. Fanslow, F.D. Finkelman, C.B. Thompson, and G. Nunez. 1996. *bcl-x* exhibits regulated expression during B cell development and activation and modulates lymphocyte survival in transgenic mice. *J. Exp. Med*. 183:381–391.
26. Nakayama, K., K. Nakayama, I. Negishi, K. Kuida, Y. Shinkai, M.C. Louie, L.E. Fields, P.J. Lucas, V. Stewart, F.W. Alt, and D.Y. Loh. 1993. Disappearance of the lymphoid system in Bcl-2 homozygous mutant chimeric mice. *Science*. 261:1584–1588.
27. Motoyama, N., F. Wang, K.A. Roth, H. Sawa, K. Nakayama, K. Nakayama, I. Negishi, S. Senju, Q. Zhang, S. Fujii, and D.Y. Loh. 1995. Massive cell death of immature hematopoietic cells and neurons in Bcl-x-deficient mice. *Science*. 267:1506–1510.
28. Choi, M.S.K., M. Holman, C.J. Atkins, and G.B.B. Klaus. 1996. Expression of *bcl-x* during mouse B cell differentiation and following activation by various stimuli. *Eur. J. Immunol*. 26:676–682.
29. Kelsoe, G. 1995. In situ studies of the germinal center reaction. *Adv. Immunol*. 60:267–288.
30. Tuscano, J.M., K.M. Druey, A. Riva, J. Pena, C.B. Thompson, and J.H. Kehrl. 1996. Bcl-x rather than Bcl-2 mediates CD40-dependent centrocyte survival in the germinal center. *Blood*. 88:1359–1364.
31. Han, S., B. Zheng, D.G. Schatz, E. Spanopoulou, and G. Kelsoe. 1996. Neoteny in lymphocytes: Rag-1 and Rag-2 expression in germinal center B cells. *Science*. 274:2092–2094.
32. Jacob, J., R. Kassir, and G. Kelsoe. 1991. In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl) acetyl. I. The architecture and dynamics of responding cell populations. *J. Exp. Med*. 173:1165–1175.
33. Zheng, B., S. Han, Q. Zhu, R. Goldsby, and G. Kelsoe. 1996. Alternative pathways for the antigen-specific selection of peripheral T cells. *Nature*. 384:263–266.
34. Jacob, J., J. Przylepa, C. Miller, and G. Kelsoe. 1993. In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. III. The kinetics of V region mutation and selection in germinal center B cells. *J. Exp. Med*. 178:1293–1307.
35. Gu, H., D. Tarlinton, W Muller, K. Rajewsky, and I. Forster. 1991. Most peripheral B cells in mice are ligand selected. *J. Exp. Med*. 173:1357–1371.
36. Han, S., S.R. Dillon, B. Zheng, M.S. Schlissel, and G. Kelsoe. 1997. V(D)J recombinase activity in a subset of germinal center B lymphocytes. *Science*. 278:301–305.
37. Nie, X., S. Basu, and J. Cerny. 1997. Immunization with immune complex alters the repertoire of antigen-reactive B cells in the germinal centers. *Eur. J. Immunol*. 27:3517–3525.
38. Manz, R.A., M. Lohning, G. Cassese, A. Thiel, and A. Radbruch. 1998. Survival of long-lived plasma cells is independent of antigen. *Int. Immunol*. 10:1703–1711.
39. Slifka, M.K., R. Antia, J.K. Whitmire, and R. Ahmed. 1998.

Humoral immunity due to long-lived plasma cells. *Immunity*. 8:363–372.
40. Han, S., K. Hathcock, B. Zheng, T. Kepler, R. Hodes, and G. Kelsoe. 1995. Cellular interaction in germinal centers. The roles of CD40-ligand and B7-2 in established germinal centers. *J. Immunol*. 155:556–567.
41. Hande, S., E. Notidis, and T. Manser. 1998. Bcl-2 obstructs negative selection of autoreactive hypermutated antibody V regions during memory B cell development. *Immunity*. 8:189–198.
42. Hartley, S.B., M.P. Cooke, D.A. Fulcher, A.W. Harris, S. Cory, A. Basten, and C.C. Goodnow. 1993. Elimination of self-reactive B lymphocytes proceeds in two stages: arrested development and cell death. *Cell*. 72:325–335.
43. Fang, W., B.C. Weintraub, B. Dunlap, P. Garside, K.A. Pape, M.K. Jenkins, C.C. Goodnow, D.L. Mueller, and T.W. Behrens. 1998. Self-reactive B lymphocytes overexpressing bcl-x_L escape negative selection and are tolerized by clonal anergy and receptor editing. *Immunity*. 9:35–45.
44. Garside, P., E. Ingulli, R.R. Mercia, J.G. Johnson, R.J. Noelle, and M.K. Jenkins. 1998. *Science*. 281:96–99.
45. Fischer, M.B., S. Goerg, L. Shen, A.P. Prodeus, C.C. Goodnow, G. Kelsoe, and M.C. Carroll. 1998. Dependence of germinal center B cells on expression of CD21/CD35 for survival. *Science*. 280:582–585.
46. Legler, D.F., M. Loetscher, R.S. Roos, I. Clark-Lewis, M. Baggiolini, and B. Moser. 1998. B cell–attracting chemokine 1, a human CXC chemokine expressed in lymphoid tissues, selectively attracts B lymphocytes via BLR1/CXCR5. *J. Exp. Med*. 187:655–660.
47. Gunn, M.D., V.N. Ngo, K.M. Ansel, E.H. Ekland, J.G. Cyster, and L.T. Williams. 1998. A B-cell-homing chemokine made in lymphoid follicles activates Burkitt's lymphoma receptor-1. *Nature*. 391:799–803.
48. Gray, D., and H. Skarvall. 1988. B-cell memory is short-lived in the absence of antigen. *Nature*. 336:70–73.
49. Schittek, B., and K. Rajewsky. 1990. Maintenance of B-cell memory by long-lived cells generated from proliferating precursors. *Nature*. 346:749–751.
50. Gauthier, E.R., L. Piche, G. Lemieux, and R. Lemieux. 1996. Role of bcl-x_L in the control of apoptosis in murine myeloma cells. *Cancer Res*. 56:1451–1456.
51. Krajewski, S., M. Krajewska, A. Shabaik, H.G. Wang, S. Irie, L. Fong, and J.C. Reed. 1994. Immunohistochemical analysis of in vivo patterns of *bcl-x* expression. *Cancer Res*. 54: 5501–5507.