Relaxed Negative Selection in Germinal Centers and Impaired Affinity Maturation in bcl-xL Transgenic Mice

By Yoshimasa Takahashi,* Douglas M. Ceracoli,* Joseph M. Dal Porto,* Michiko Shimoda,* Robert Freund,* Wei Fang,‡ David G. Telander,* Erika Nell M. Alvey,‡ Daniel L. M. Uller,‡ Timothy W. Behrens,‡ and Garnett Kelsoe*‡

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-xL, in the B cell compartment. Although transgenic animals briefly expressed higher numbers of splenic AFCs after immunization, the bcl-xL 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-xL transgene product, in addition to endogenous Bcl-xL, 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 G1 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-xL and apoptosis influence clonal selection/maintenance for affinity maturation.

Key words: Bcl-xL • 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) 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 (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-
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 bd-2, bd-x, also suppresses apoptosis through its Bcl-xL product (23). Bcl-xL 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-xL is important for the survival of immature lymphocytes (27). Interestingly, cross-linking of mlg or CD40 on splenic B cells upregulates the expression of Bcl-xL (25, 28). Human GC B cells are also known to reexpress Bcl-xL, 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-xL might control life-or-death decisions in the GC compartment.

To examine the roles of apoptosis and Bcl-xL in affinity maturation, we tracked the affinity of antibody-forming cells (AFCs) in the bone marrow (BM) and serum antibody of bd-x1 transgenic mice and their congenic wild-type controls, during the clonally restricted antibody response to the (4-hydroxy-3-nitropheryl)acetyl (N P) hapten (32). Our study revealed that overexpression of Bcl-xL 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 bd-x1 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 antibodies that are normally rare in the later stages of the primary anti-NP response and generally encode lower-affinity antibodies. 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.
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-FcRγII and then stained with biotinylated anti-IgD, -M–a-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 and TR-FITC-labeled anti-CD3 and PE-conjugated anti-B220 antibody, 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

bd-x<sub>L</sub> Transgene Protects B Cells from Passive A apoptotic Death. Spleens of mice carrying the bd-x<sub>L</sub> 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<sup>+</sup> B220<sup>+</sup> 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.
Proteins are abundant in the follicular population of mice carrying the bd-\(x\) transgene. In contrast, GC B cells (GL-7\(^+\) B220\(^+\)) isolated from both wild-type and transgenic mice abundantly express Bcl-\(x\) but little or no Bcl-2. This observation is consistent with studies of GCs in humans (18, 30). Thus, the reciprocal expression of Bcl-\(x\) and Bcl-2 was observed in pre-B cells holds for GC B cells (24, 25). Interestingly, although transgenic Bcl-\(x\) is strongly expressed in follicular B cells, only modest amounts of tagged Bcl-\(x\) could be demonstrated in GC B cells. Reverse transcription PCR studies confirm lower steady state levels of transgenic Bcl-\(x\) message in the GL-7\(^+\) B220\(^+\) cell population (data not shown). This biased expression of transgenic Bcl-\(x\) may represent distinct E\(_{\text{B}}\) activity in each B cell compartment (the density of mlg on GC B cells is \(\leq\)10% of that found on follicular B cells [29]) or downregulation of the transgene's herpes TK promoter in activated cells (24).

GCs develop normally in bd-\(x\) transgenic mice. To assess the effects of the bd-\(x\) 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 \(\lambda\) L chain and an H chain encoded by a canonical VDJ gene rearrangement (32). We identified \(\lambda\)\(^+\) GCs in spleen sections by labeling with PNA and anti-\(\lambda\) antibody (32) and determined the average number of \(\lambda\)\(^+\) 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 \(\lambda\)\(^+\) 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 \(\lambda\)\(^+\) GCs was 7.6%. Thus, frequent \(\lambda\)\(^+\) GCs in transgenic mice result from immunization with NP rather than altered \(\lambda\) L chain expression.

The frequencies of splenic GC B cells (GL-7\(^+\) B220\(^+\)) 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 ± 24% of PNA\(^+\) GC cells were labeled by a 2-h pulse of BrdU (not shown).

Transgenic Bcl-\(x\) Increases Splenic AFC Numbers but not Their Longevity or Serum Antibody Titers. T cell–dependent antigens induce two distinctive 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 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 2.** Expression of endogenous and transgenic Bcl-\(x\) in splenocytes. Expression patterns of Bcl-\(x\) and Bcl-2 were determined by Western blotting of splenocytes from bd-\(x\) transgenic mice or wild-type littermate controls. (A) Unselected (total splenic [Total spl], lane 1), lymphoid gate (lane 2), and sorted (B220\(^+\)CD3\(^-\), lane 3; B220\(^+\)CD3\(^+\), lane 4) splenocytes were recovered from naïve transgenic mice. Most, if not all, transgenic Bcl-\(x\) 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 \(\mu\)g of cell lysate protein was immunoblotted using anti-Bcl-x (upper panel) or anti-Bcl-2 (lower panel) mAbs. Molecular masses: transgenic Bcl-\(x\), 33 kD; endogenous Bcl-\(x\), 31, 32 kD; Bcl-2, 26 kD. Transgenic Bcl-\(x\) protein is abundant in follicular (GL-7\(^+\) B220\(^+\)) B cells, but only modest amounts are present in GC B lymphocytes (GL-7\(^+\) B220\(^-\)). Endogenous Bcl-\(x\) 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.
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-xL 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.

**TUNEL assays** on spleen sections from transgenic and control mice to determine if the small addition of transgenic Bcl-xL 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-xL 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-xL transgenics. Perhaps more significantly, >20% of GCs in control mice contained >3 TUNEL + cells/unit area, whereas only 5% of GCs in bcl-xL 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-xL 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-xL transgenic mice. Initially, immuni-
Relaxed Affinity Maturation in bcl-xL Transgenic Mice

404

zation with NP conjugates stimulates a broad population of splenic B cells that bear the \( \lambda_1 \) 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 VH 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 VH 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 VH genes.

Figure 4. bcl-xL 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 one of 12 categories. The distributions of categories for transgenic and wild-type controls are plotted.

Table I. Somatic Genetics of \( \lambda_1^+ \) GC Cells in bcl-xL Transgenic and Wild-type Mice 12 d after Immunization

|                | bd-xL | 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 (bd-xL) and AF065332-42 (wild-type).

*R/S ratio of V_H V186.2 given random mutagenesis.

‡Percentage of rearrangements using DFL16.1 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.
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 bd-\(x_L\) 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 a population (53%) of mice with the noncanonical VDJ rearrangements present in day 12 GCs joined to C\(\gamma 1\). Amplified VDJ rearrangements were cloned and sequenced to identify the V\(H\) and D gene segments used and any mutations present. Table I summarizes this work and shows that only half (11/21) of the VDJ sequences recovered from \(bd-x_L\) 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 \(bd-x_L\) transgene. The reduced average affinity of BM AFCs in \(bd-x_L\) transgenic mice results from the retention of B cells bearing noncanonical VDJ rearrangements. Interclonal competition in both 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 \(\approx 60\%\) of controls. Overexpression of Bcl-\(x_L\) led to diminished affinity maturation in both BM AFCs and the serum antibody.

**Figure 5.** \(bd-x_L\) transgenic mice show relaxed affinity maturation of NP-specific IgG1 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 IgG1 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 IgG1 antibody were determined by ELISA, and the ratios of NP\(_2\) versus NP\(_{23}\)-specific IgG1 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\textsubscript{L} Transgenic and Wild-type Mice 69 d after Immunization

|                   | bcl-x\textsubscript{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          |                         |           |
| CDR 1 (14.0/1)*    | 1.8/1                   | 14.0/1    |
| CDR 2 (4.3/1)      | 3.1/1                   | 8.2/1     |
| FW (3.1/1)         | 2.1/1                   | 1.5/1     |
| DFL16.1 (% of total)\textsuperscript{b} | 46 | 69 |
| YYGS (% of total)\textsuperscript{a} | 27 | 44 |
| W→L33 (% of total)\textsuperscript{c} | 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\textsubscript{L}) and AF065364-80 (wild-type).

* R/S ratio of V\textsubscript{H} V186.2 given random mutagenesis.
\textsuperscript{a} Percentage of rearrangements using DFL16.1 gene segments in all rearrangements of V\textsubscript{H} V186.2.
\textsuperscript{b} Percentage of rearrangements encoding YYGS in CDR3 in all rearrangements of V\textsubscript{H} V186.2.
\textsuperscript{c} Percentage of all V\textsubscript{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\textsubscript{L} expression. The average frequency of mutations in V186.2 V\textsubscript{H} gene segments from the BM AFCs of bcl-x\textsubscript{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 CDR 1 and CDR 2 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\textsubscript{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\textsubscript{2a} 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\textsubscript{L} also retards affinity maturation outside the GC microenvironment.

Discussion

In this report, we have demonstrated that a bcl-x\textsubscript{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\textsubscript{L} activity; although Bcl-x\textsubscript{L} is abundant in the GL-7\textsuperscript{+}B220\textsuperscript{+} 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\textsubscript{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\textsubscript{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\textsubscript{L} becomes abundant in B cells after BCR (24, 30). Bcl-x\textsubscript{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\textsubscript{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\textsubscript{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\textsubscript{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-xL 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-xL 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-xL transgenic mice, we think it is unlikely that Bcl-xL 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-xL 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-xL 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 only on Bcl-xL (51). Alternatively, selective competition among BM AFCs for antigen might drive sustained affinity maturation. BM AFCs express low levels of mIg and could interact with antigen dependently in a Bcl-xL-dependent fashion. Indeed, plasma cytoplasms express Bcl-xL (50), and human plasma cells exhibit high levels of Bcl-xL 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 AR 43805 (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-

5. Takahashi et al.
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. Abbott, 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.R. ajewsky, and U. Weiss. 1991. Intrac nal selection 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. Cél. 67:1121–1129.

10. Smith, K.G.C., A. Light, G.J.V. Nossal, and D.M. Tarlinton. 1995. The extent of affinity maturation differs between the memory and antibody-forming cell compartments in the primary immune response. EMBO (Eur. Mol. Biol. Org.) 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. 187:885–931.

12. Dal Porto, J., A. Herman, M. Shlomchik, and G. Kelsoe. 1998. Antigen drives very low affinity B cells to become plasma cells and enter germinal centers. J. Immunol. 161:5373–5381.

13. Batista, F.D., and M.S. Nueberger. 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. Kanourakis, 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 centres 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, P53, 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. Durant, 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. Immunology. 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. R ajewsky, 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.
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. Baeten, 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-xL 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. Noel, 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. Baggioili, 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-xL 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.