Transgenic Expression of Bcl-x$_L$ or Bcl-2 by Murine B Cells Enhances the In Vivo Antipolysaccharide, but Not Antiprotein, Response to Intact *Streptococcus pneumoniae*[1,2]

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IgG antipolysaccharide (PS) and antiprotein responses to *Streptococcus pneumoniae* (Pn) are both CD4$^+$ T cell dependent. However, the primary IgG anti-PS response terminates more quickly, uses a shorter period of T cell help, fails to generate memory, and is more dependent on membrane Ig (mIg) signaling. We thus determined whether this limited anti-PS response to Pn reflected a greater propensity of PS-specific B cells to undergo apoptosis. We used mice that constitutively expressed the antiapoptotic protein Bcl-x$_L$ or Bcl-2 as a B cell-specific transgene. Both transgenic (Tg) mice exhibited increased absolute numbers of splenic B-1 and peritoneal B-1b and B-2 cells, subsets implicated in anti-PS responses, but not in marginal zone B (MZB) cells. Both Tg mouse strains elicited, in an apparently Fas-independent manner, a more prolonged and higher peak primary IgM and IgG anti-PS, but not antiprotein, response to Pn, but without PS-specific memory. A similar effect was not observed using purified PS or pneumococcal conjugate vaccine. In vitro, both splenic MZB and follicular Tg B cells synthesized DNA at markedly higher levels than their wild-type counterparts, following mIg cross-linking. This was associated with increased clonal expansion and decreased apoptosis. Using Lsc$^{-/-}$ mice, the Pn-induced IgG response specific for the capsular PS was found to be almost entirely dependent on MZB cells. Collectively, these data suggest that apoptosis may limit mIg-dependent clonal expansion of PS-specific B cells during a primary immune response to an intact bacterium, as well as decrease the pool of PS-responding B cell subsets. *The Journal of Immunology*, 2007, 179: 7523–7534.

P urified soluble polysaccharide (PS),[5] in contrast to protein, Ags can generate comparable Ig responses in wild-type (WT) and T cell-deficient hosts, and are thus referred to as T cell-independent (TI) Ags (1). This, in part, reflects the inability of PS Ags to associate with MHC class II molecules, precluding the recruitment of cognate CD4$^+$ T cell help (2, 3). Zwitterionic PS represent a notable exception, but constitute only a minor subgroup of all PS found in nature (4, 5). When a PS Ag is covalently linked to an immuno-

genic protein (i.e., conjugate vaccine), it is transformed into a classical T cell-dependent (TD) Ag. Thus, uptake of the conjugate, and processing of the protein component, by the PS-specific B cell leads to presentation of peptide-MHC class II to specific CD4$^+$ T cells (6, 7). As a result, anti-PS responses to conjugate vaccines, in contrast to isolated PS Ags, but similar to isolated proteins, exhibit more prolonged primary kinetics of induction, class switching to IgG isotypes in addition to IgG3, and the generation of PS-specific memory.

Typically, however, the host encounters PS Ags in the context of their expression by an intact microorganism. Thus, in nature, PS Ags are initially presented to the immune system within a particulate structure, associated with numerous distinct proteins, as well as ligands for various innate immune cell receptors that can regulate cellular recognition, uptake, and activation. PS in this context are therefore potentially distinct immunogens and hence may elicit Ig responses that are governed by mechanisms that differ from those observed using simplified model Ags. Indeed, accumulating evidence from our laboratory indicates that PS Ags expressed by intact bacteria may be neither classically TI nor TD (8). Thus, immunization of mice with intact *Streptococcus pneumoniae* (Pn), in contrast to an isolated pneumococcal PS Ag, elicits an anti-PS response comprising all four IgG isotypes, that is dependent on CD4$^+$ T cell help and B7-dependent costimulation, similar to that observed for the concomitant IgG antiprotein response (9, 10). However, unlike the antiprotein response, the IgG anti-PS response to intact Pn exhibits a significantly shortened period of primary induction and no apparent memory upon secondary immunization. This is associated with a substantially shorter period of dependence on CD4$^+$ T cell help (10) and B7-dependent costimulation (11) for the primary IgG anti-PS, vs antiprotein, response and a greater dependence on B cell membrane Ig (mIg) signaling, mediated by Bruton's tyrosine kinase (Btk) (12).

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Thus, the basis for the relative limitation of the IgG anti-PS, relative to the antiprotein response to intact Pn, is not critically related to the absolute ability to recruit CD4$^+$ T cell help (i.e., TI vs TD nature of the Ags). In this regard, the strength, duration, and/or relative dependence of the mIg signal upon Ag binding, the relative efficiency in recruiting CD4$^+$ T cell help, and the involvement of distinct B cell subsets may be more important considerations. One potential consequence is that, collectively, these parameters could impact differentially upon the survival/apoptosis of PS- vs protein-specific B cells, accounting in part for the different primary kinetics and level of induction of the PS-specific Ig response, and perhaps in the failure to generate PS-specific memory.

Two proteins that play a major role in promoting B cell survival are Bcl-x$_L$ and Bcl-2. Although these proteins appear to be equipotent in promoting B cell survival and may act through similar mechanisms (13), they are expressed at different stages of B cell development and activation. Bcl-2 appears to be critical for mature B cell survival (14), whereas Bcl-x$_L$ may be more important in promoting viability of proliferating (immature and activated) B cells (15). Thus, resting peripheral B cells constitutively express Bcl-2, whereas Bcl-x$_L$, although not Bcl-2, is up-regulated in these cells upon mIg cross-linking, CD40 signaling, or LPS stimulation resulting in their coexpression (16). Bcl-2 is then down-regulated in B cells within the germinal center (GC), the key site for the generation of memory B cells (17), where extensive apoptosis occurs (18, 19). However, in vitro stimulation via membrane Ig or CD40 promotes GC B cell viability, associated with Bcl-2 up-regulation (20). Additionally, the survival of high-affinity memory B cells can be maintained by Bcl-2 expression (21). Bcl-x$_L$, in contrast to Bcl-2, is typically expressed in freshly isolated GC (centrocyte) B cells (22, 23). In light of these observations, we used mice that constitutively expressed transgenic Bcl-x$_L$ or Bcl-2, selectively within B cells, to ask whether the sustained activity of either of these proteins could differentially impact on primary and/or secondary anti-PS and/or antiprotein Ig responses to intact Pn, as well as a soluble pneumococcal conjugate vaccine, or isolated pneumococcal PS.

Materials and Methods

Mice

B cell-specific Bcl-x$_L$ transgenic (Tg) mice provided by T. Behrens (University of Minnesota, Minneapolis, MN) were originally made using the human Bcl-x$_L$-Tg cDNA cloned into an expression vector under the regulatory control of the SV40 promoter and IgH enhancer (16), and were backcrossed 11 times onto BALB/c mice. B cell-specific Bcl-2-Tg mice were similarly made using a human Bcl-2 cDNA inserted into an expression vector containing the IgH enhancer and SV40 promoter (24) and were backcrossed 20 times onto BALB/c mice. Female and male Bcl-x$_L$ and Bcl-2-Tg mice were used between 10 and 16 wk of age. Gender and age-matched WT BALB/c mice (The Jackson Laboratory) were used as controls in all experiments. The experiments were conducted under the National Cancer Institute Animal Study Protocols LG023, LG024, and LG025. Mice were genotyped by PCR using the following primers: 1) Bcl-x$_L$ Tg: sense: 5′-GGCGGGCATTCAGTGACCTG-3′; antisense: 5′-TGAGCCCAGCAGAACCACGCCG-3′; bands: 396-bp transgenic bcl-x$_L$, 321-bp endogenous bcl-x$_L$; 2) Bcl-2-Tg: sense, GCAGACACTCTATGC CTGTGTGG; antisense: GGAACTGATGAATGGGAGCAGT; bands: 361-bp transgenic Bcl-2, no band for endogenous Bcl-2. Lsc$^{-/-}$ mice (25), used between 8 and 12 wk of age, were generated in, and bred on, a C57BL/6 genetic background, and bred and maintained at the National Jewish Biological Resource Center (Denver, CO). Female lpr (Fas-defective), gld (FasL-defective), and WT (C3H/HeJ) mice were purchased from The Jackson Laboratory and were used at 8 wk of age.

Reagents

Recombinant pneumococcal surface protein A (PspA) was expressed in Saccharomyces cerevisiae BJ3505 and purified as previously described (26). Purified PspA was >95% pure by Coomassie blue staining. PC-key-hole limpet hemocyanin (KLH) was synthesized as described previously (9). The resulting conjugate had a substitution degree of 19 PC/KLH. Purified Pn capsular polysaccharide type 14 (PPS14) was purchased from the American Tissue Culture Collection. Purified Pn cell wall C-PS was purchased from Statens Serum Institut. The soluble C-PS-PspA and PPS14-PspA conjugates were synthesized as previously described (10). Dextran-conjugated anti-IgD (αδ-dex) was prepared by conjugation of an "a" allotype-specific anti-IgD mAb (clone Hδ^a/1) to dextran (2×10^6 m.w.) (27). Agonistic Armenian hamster IgM, κ anti-mouse CD40 mAb (clone HM40-3, no azide, low endotoxin) was obtained from BD Biosciences. LPS from Escherichia coli serotype 0111:B4 was purchased from Sigma-Aldrich. Goat F(ab′)$_2$ anti-mouse IgM was purchased from Southern Biotechnology Associates.

Preparation and immunization of Pn, capsular type 14

A frozen stock of Pn, capsular type 14 was thawed and subcultured on BBL premade blood agar plates (VWR International). Isolated colonies in blood agar were grown in Todd Hewitt broth (BD Biosciences) to mid-log phase, collected, and heat killed by incubation at 60°C for 1 h. Sterility was confirmed by subculture on blood agar plates. After extensive washings, the bacterial suspension was adjusted with PBS to give an absorbance reading at 650 nm of 0.6 which corresponded to 10^9 CFU/ml. Bacteria were then aliquoted at 10^{10} CFU/ml and frozen at -80°C until their use as Ag for mouse immunizations.

Immunizations

Mice were immunized i.p. with 2×10^8 CFU heat-killed Pn14 in saline or PPS14-PspA plus C-PS-PspA (conjugates) adsorbed on 13 μg of alum (allhydrogel 2%; Brenntag Biosector) mixed with 25 μg of a stimulatory 30-mer CpG-containing oligodeoxynucleotide (CpG-ODN) (28), and similarly boosted. One microgram each of purified C-PS and PPS14 was injected i.p. in saline. Serum samples for measurement of anti-PPS14, anti-PC, and anti-PspA Ig isotype titers were prepared from blood obtained through the tail vein.

Measurement of serum Ig isotype titers of anti-PC, anti-PPS14, and anti-PspA

ELISA plates were coated with 5 μg/ml (50 μl/well) of PC-KLH (Immulon 2 plates; Dynex Technologies), PPS14 (Immulon 2 or 4), or PspA (Immulon 4) in PBS for 1 h at 37°C or overnight at 4°C. Plates were washed three times with PBS plus 0.1% Tween 20 and were blocked with PBS plus 1% BSA for 30 min at 37°C or overnight at 4°C. Three-fold dilutions of serum samples, starting at a 1/50 serum dilution, in PBS plus 0.05% Tween 20 were then added for 1 h at 37°C or overnight at 4°C and plates were washed three times with PBS plus 0.1% Tween 20. Alkaline phosphatase-conjugated polyclonal goat anti-mouse IgM, IgG, and IgG isotype Abs (200 ng/ml final concentration in PBS plus 0.05% Tween 20) were then added, and plates were incubated for 37°C for 1 h. Plates were washed five times with PBS plus 0.1% Tween 20. Substrate (p-nitrophenyl phosphate, disodium; Sigma-Aldrich) at 1 mg/ml in TM buffer (1 M Tris plus 0.3 mM MgCl$_2$ (pH 9.8)) was then added for 30 min at room temperature for color development. Color was read at an absorbance of 405 nm on a Multiskan Ascent ELISA reader (Labsystems).

Purification of splenic B cells

Single-cell suspensions from spleen were prepared, and RBCs were lysed using ACK lysing buffer (Quality Biological). B cells were positively selected by magnetic bead sorting using anti-mouse CD45R (B220) micromagnetic beads (Miltenyi Biotec) according to the manufacturer's instructions. Cell purities were checked by flow cytometry following each purification and found to be 90–92% B220$^+$ cells. Purified B cells were used immediately for cell culture studies.

Measurement of DNA synthesis by [^{3}H]TdR incorporation

Purified B220$^+$ splenic B cells were cultured (2.5×10^5 cells/ml in 0.2 ml) in medium (RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 0.05 mM 2-ME, 50 μg/ml penicillin, and 50 μg/ml streptomycin), in the presence of various stimuli, in flat-bottom 96-well Costar plates (Corning). After various times in culture at 37°C in a 5% CO$_2$-containing incubator, [^{3}H]TdR (2 μCi; specific activity of 25 Ci/mM or 925 GBq/mM, catalog no.TRK120; Amersham Biosciences) was added to the cultures for an additional 18 h. Cultured cells were then harvested onto glass filter paper (catalog no. 1450-421; Wallac) using a Harvester 96 (Tomtec). Specific incorporation of [^{3}H]TdR was determined using a 1450 Microbeta, "Wallac" Trilux scintillation counter.

Measurement of cell division by CFDA-SE dilution

A total of 2.5×10^7 cells were pelleted and washed with PBS containing 0.1% BSA (buffer), resuspended in 1 ml of buffer, and incubated at 37°C with 5 μM final concentration of carboxy-fluorescein diacetate, succinimidyl ester (Vybrant CFDA-SE; Molecular Probes) for 10 min. After labeling, cells were washed two times with RPMI 1640 plus 10% FBS and resuspended in medium. CFDA-SE-loaded B cells were cultured for varying times at 5×10^5 cells/ml in 24-well plates (2 ml/well) in the presence of $\alpha\delta$-dex (10 ng/ml final concentration). Cells were analyzed using a Coulter Epics XL-MCL flow cytometer (Beckman Coulter) and "proliferation index" (PI) based on CFDA-SE dilution was obtained by using Modfit software (Verity Software House). Specifically, the PI is the sum of the cells in all generations divided by the calculated number of original parent cells.

Measurement of apoptotic cells using propidium iodide

B cells undergoing apoptosis were identified by their reduced relative nuclear DNA content using propidium iodide incorporation in a hypotonic solution followed by flow cytometric analysis as previously described (29). Briefly, B cells were cultured for 4 days at 5×10^5 cells/ml in 24-well plates (2 ml/well) in the presence of $\alpha\delta$-dex (10 ng/ml final concentration). B cells were then resuspended in a hypotonic solution containing 50 μg/ml PI (Molecular Probes), 0.1% Triton X-100, and 0.1% sodium citrate. Samples were stored at 4°C for 16–18 h in the dark and vortexed before analysis using a Coulter Epics XL-MCL flow cytometer. Data were analyzed using "Winlist" software (Verity Software House).

Flow cytometric analysis and electronic cell sorting

Splenic and peritoneal cells from WT and Tg mice (three mice per group, each stained separately) were harvested, and B cell subsets were enumerated by flow cytometry as follows: splenic marginal zone B (MZB) (CD21^{high}CD23$^{low/neg}$), follicular B (FB) (CD21^{int}CD23^{high}), and B-1 (B220^{intermed}CD5$^+$), peritoneal B-1a (B220$^+$CD11b$^+$CD5$^+$), B-1b (B220$^+$CD11b$^+$CD5$^-$), and B-2 (B220$^+$CD11b$^-$CD5$^-$). The following mAbs, purchased from BD Pharmingen, were used: FITC-rat IgG2b,κ anti-mouse CD21/35 (clone 7G6) PE-rat IgG2a,κ anti-mouse CD23 (clone B3B4), FITC-rat IgG2a,κ anti-mouse CD45R/B220 (clone RA3-6B2), biotin-rat IgG2a,κ anti-mouse CD5 (Ly-1; clone 53–7.3) plus streptavidin-PE-Texas Red, and PE-rat IgG2b,κ anti-mouse CD11b (clone M1/70). Cells were analyzed on a LSR-II flow cytometer (BD Biosciences) using 488 and 635 lasers and results were generated using Winlist software. For isolation of MZB and FB cells, RBC-lysed spleen cells were stained for these B cell subsets as above, and purified using a BD Biosciences FACSAria flow cytometer cell sorter. Purities of >98% for each B cell subset were obtained.

Statistical analysis

Data are expressed as geometric mean ± SEM. Significance (*; $p < 0.05$) between groups was determined using the Student t test.

Results

Bcl-x$_L$-Tg and Bcl-2-Tg mice exhibit significant increases in splenic B-1 and peritoneal B-1b cells

Because B cell subsets may contribute differentially to anti-PS and antiprotein Ig responses, we first wished to determine the percentages and absolute numbers of these cells in the spleen and peritoneum of Bcl-x$_L$-Tg and Bcl-2-Tg mice, relative to WT mice. Both Tg mouse strains exhibited significant ($p < 0.05$) increases in the total number of spleen cells (Bcl-x$_L$-Tg: 2.5-fold, Bcl-2-Tg: 2.5-fold) and peritoneal cells (Bcl-x$_L$-Tg: 5.5-fold, Bcl-2-Tg: 3.8-fold), reflecting in large part, an increase in total B cells (Table I). Although the percentages of B-1 cells were similar in WT and Tg mice, the absolute number of B-1 cells was significantly increased (Bcl-x$_L$-Tg: 2.8-fold, Bcl-2-Tg: 3.0-fold). In contrast, the percentages of splenic MZB cells in Tg mice were significantly reduced (Bcl-x$_L$-Tg: 3.2-fold, Bcl-2-Tg: 4.4-fold) but the absolute numbers were not statistically different relative to WT mice. Neither the percentages nor absolute numbers of splenic FB cells were significantly different in WT and Tg mice. Although the percentages of peritoneal B-1b cells in Tg mice were only modestly higher than in WT mice, the absolute numbers were substantially increased (Bcl-x$_L$-Tg: 8.4-fold, Bcl-2-Tg: 6.1-fold). Similarly, the absolute numbers of peritoneal B-2 cells were strikingly higher in Tg mice (Bcl-x$_L$-Tg: 12-fold, Bcl-2-Tg: 16-fold) whereas the percentages were more modestly increased (Bcl-x$_L$-Tg: 2.2-fold, Bcl-2-Tg: 3.2-fold). In contrast, no significant differences were observed between WT and Tg mice in the absolute numbers of peritoneal B-1a cells, whereas the percentages were significantly reduced in Tg mice (Table I). Thus, B cell subsets known to play a selective role in TI anti-PS responses (splenic B-1 cells (30, 31), peritoneal B-1b cells (32, 33), and possibly peritoneal B-2 cells (34)) are elevated in Tg mice, whereas those implicated in antiprotein responses (FB cells) are similar between WT and Tg mice.

Bcl-x$_L$-Tg and Bcl-2-Tg mice elicit higher primary anti-PS, but not antiprotein, responses to intact Pn, relative to WT control mice

We next wished to determine whether Tg mice exhibit differences in their elicitation of primary and/or secondary anti-PS and/or antiprotein Ig isotype responses to intact Pn, capsular type 14 (Pn14)). Bcl-x$_L$-Tg and Bcl-2-Tg mice were immunized i.p. with heat-killed Pn14 suspended in saline, and boosted in a similar fashion 14 days later. Sera were obtained at various time points to determine, by ELISA, the relative serum titers of IgM and/or IgG specific for the type 14 capsular PS (PPS14), the phosphorylcholine (PC) determinant of the C-polysaccharide (teichoic acid), and the cell wall, PC-binding protein, PspA. As illustrated in Fig. 1*A*, Bcl-x$_L$-Tg mice exhibited a significant ($p < 0.05$) 4.1- and 9.0-fold increase in peak primary serum titers of IgM and IgG anti-PPS14, respectively, relative to WT mice. A similar significant 6.9- and 2.5-fold increase in the peak primary IgM and IgG anti-PPS14 responses was also observed in Bcl-2-Tg mice (Fig. 1*B*).

Table I. *B cell subsets in WT, Bcl-x$_L$, and Bcl-2-Tg mice*

| | | %[a] (Total 10^6)[b] | | |
| | | Cell type | | |
Spleen	Total Cells (10^6)	FB cells	MZB cells	B-1 cells
WT	82 ± 12.4	28 ± 2.3 (23 ± 5)	4 ± 0.5[c] (3 ± 0.8)	3.7 ± 0.15 (3.1 ± 0.5)
Bcl-x$_L$	210 ± 23.1[c]	19 ± 0.9 (40±6)	1.2 ± 0.1[c] (2.4 ± 0.5)	4.3 ± 0.2 (8.8 ± 0.6)[c]
Bcl-2	200 ± 20.8[c]	19 ± 3.5 (39 ± 4)	0.9 ± 0.2[c] (1.7 ± 0.6)	4.6 ± 0.2 (9.2 ± 1.5)[c]
Peritoneum		B-1a cells	B-1b cells	B-2 cells
WT	1 ± 0.2	44 ± 2 (0.44 ± 0.08)	31 ± 0.3 (0.3 ± 0.06)	13 ± 1.7 (0.1 ± 0.03)
Bcl-x$_L$	5 ± 0.6[c]	17 ± 1.9[c] (0.95 ± 0.2)	47 ± 1.8[c] (2.6 ± 0.2)[c]	29 ± 1.7[c] (1.6 ± 0.2)[c]
Bcl-2	4 ± 1.1[c]	11 ± 0.2[c] (0.55 ± 0.1)	40 ± 0.6[c] (2 ± 0.3)[c]	42 ± 1.2[c] (2 ± 0.4)[c]

[a]Mean ± SEM of percentage of each B cell subset relative to total number of cells (three mice per group).
[b]Mean ± SEM of total number of each B cell subset calculated as the percent times total cells (10^6).
[c]Significance $p < 0.05$; Tg relative to WT.
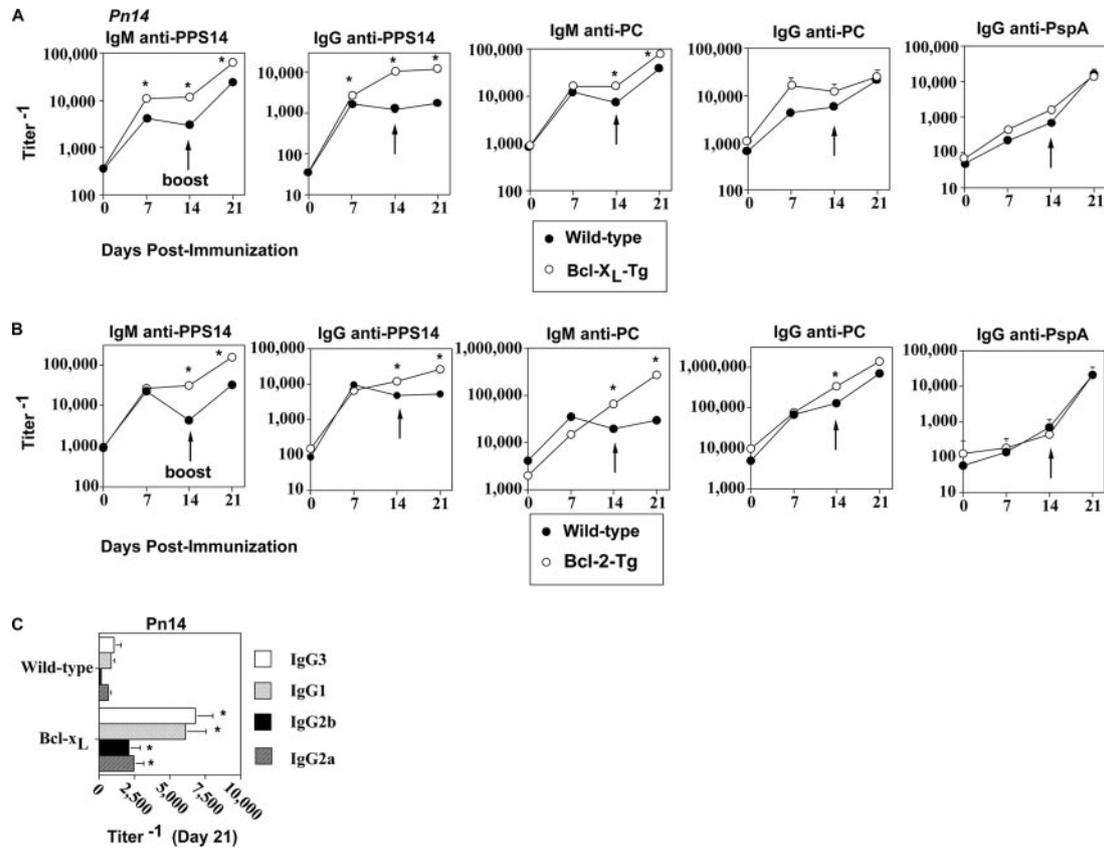
FIGURE 1. Bcl-x$_L$-Tg and Bcl-2-Tg mice elicit higher primary anti-PS, but not antiprotein responses to intact Pn, relative to WT control mice. *A* and *C*, Bcl-x$_L$-Tg and WT (BALB/c) (seven mice per group) and, *B*, Bcl-2-Tg and WT (BALB/c) (seven mice per group) mice were immunized i.p. with 2 × 10^8 CFU equivalents of heat-killed Pn14 in saline. Mice were similarly boosted on day 14. Sera were collected on the indicated days for measurement of Ag-specific IgM and IgG isotype titers. Data are presented as geometric mean ± SEM; ∗, significance $p < 0.05$. One of three similar experiments using Bcl-xL-Tg mice is shown; data on Bcl-2-Tg mice represent a single experiment.

Importantly, these increases largely reflected a more sustained primary Ig response in Tg mice. Upon secondary immunization, comparable boosts in serum IgM anti-PPS14 titers were observed in Tg and WT mice with Bcl-x$_L$-Tg (Fig. 1*A*) and Bcl-2-Tg (Fig. 1*B*) mice still showing significant 2.5- and 4.7-fold higher titers, respectively, relative to WT mice. Secondary IgG anti-PPS14 responses showed little, if any, boosting in serum titers in either Tg or WT mice, with Bcl-x$_L$-Tg (Fig. 1*A*) and Bcl-2-Tg (Fig. 1*B*) mice maintaining 6.8- and 5.0-fold higher secondary titers, respectively, relative to WT mice. Further analysis of secondary PPS14-specific IgG isotypes in Bcl-x$_L$ and WT mice demonstrated significantly ($p < 0.05$) higher titers of all IgG isotypes (IgG3 (6.3-fold), IgG1 (7.0-fold), IgG2b (14-fold), and IgG2a (3.6-fold)) in Bcl-x$_L$ relative to WT mice (Fig. 1*C*).

Primary and secondary IgM anti-PC responses were also significantly higher in Tg relative to WT mice (Bcl-x$_L$-Tg: primary 2.2-fold, secondary 2.1-fold; Bcl-2-Tg: primary 3.3-fold, secondary 9.2-fold), similar to that observed for IgM anti-PPS14 titers, although IgG anti-PC responses were largely equivalent (Fig. 1, *A* and *B*). Of note, no significant differences were observed in primary or secondary serum titers of IgG anti-PspA in Bcl-x$_L$-Tg or Bcl-2-Tg mice relative to WT mice. Similar results were observed for IgG responses specific for two additional pneumococcal proteins (pneumococcal surface protein C and pneumococcal surface adhesin A) (data not shown). These data demonstrate that forced expression of antiapoptotic proteins within B cells results in a selective enhancement in anti-PS vs antiprotein responses to intact Pn14 largely due to a prolongation of the primary response, with-

out a significant effect on the secondary response following boosting.

Anti-PS responses to PPS14-PspA plus C-PS-PspA conjugate vaccine or to purified PPS14 and C-PS are similar in Bcl-x$_L$ and WT mice

As discussed in the *Introduction*, a number of key parameters associated with the induction of the anti-PS response to intact Pn are distinctly different from those associated with the anti-PS response to conjugate vaccine or to purified PS. In this regard, the intact pathogen behaves as a relatively unique immunogen. To investigate this further, we asked whether constitutive B cell-expression of transgenic Bcl-x$_L$ had a similar enhancing affect on the anti-PS response to either a mixture of two soluble pneumococcal conjugates (PPS14-PspA plus C-PS-PspA) or to purified PPS14 plus C-PS. Thus, Bcl-x$_L$ Tg and WT mice were immunized i.p. with the conjugate mixture in the presence of adjuvant (alum plus CpG-ODN) or i.p. with the two isolated PS in saline. For the conjugates, mice were boosted on days 28 and 56, whereas for the isolated PS mice were boosted on day 14. As illustrated in Fig. 2*A*, the primary, secondary, and tertiary IgM and IgG anti-PS (anti-PPS14 and anti-PC) and antiprotein (anti-PspA) responses were similar between WT and Bcl-x$_L$ mice. Likewise, no significant differences between WT and Bcl-x$_L$-Tg mice were observed for IgM and IgG anti-PC and anti-PPS14 responses to the isolated PS (Fig. 2*B*). These data further support the notion that the anti-PS response to intact Pn14 exhibits unique features relative to that observed following immunization with either conjugate vaccine or isolated PS.
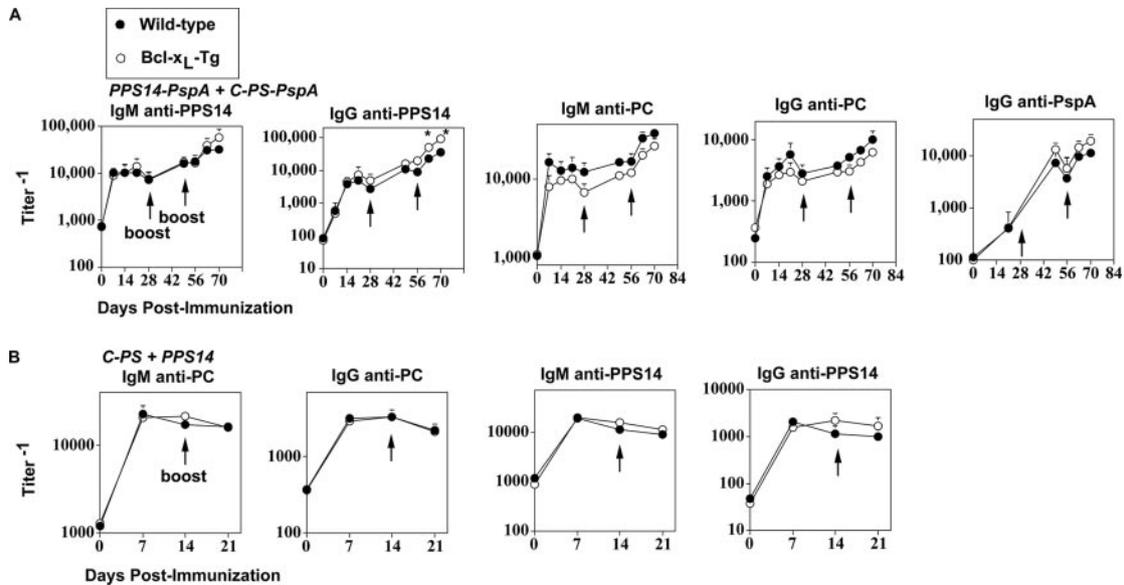
FIGURE 2. Anti-PS responses to PPS14-PspA + C-PS-PspA conjugate vaccine or to purified PPS14 and C-PS are similar in Bcl-x$_L$ and WT mice. *A*, Bcl-x$_L$-Tg mice (seven mice per group) were immunized i.p. with 1 μg each of PPS14-PspA and C-PS-PspA suspended in alum + CpG-ODN and similarly boosted on days 28 and 49. *B*, Bcl-x$_L$-Tg mice (seven mice per group) were immunized i.p. with 1 μg each of C-PS and PPS14 in saline and similarly boosted on day 14. Sera were collected on the indicated days for measurement of Ag-specific IgM and IgG isotype titers. Data are presented as geometric mean ± SEM; *, significance $p < 0.05$. One of two similar experiments is shown for both *A* and *B*.
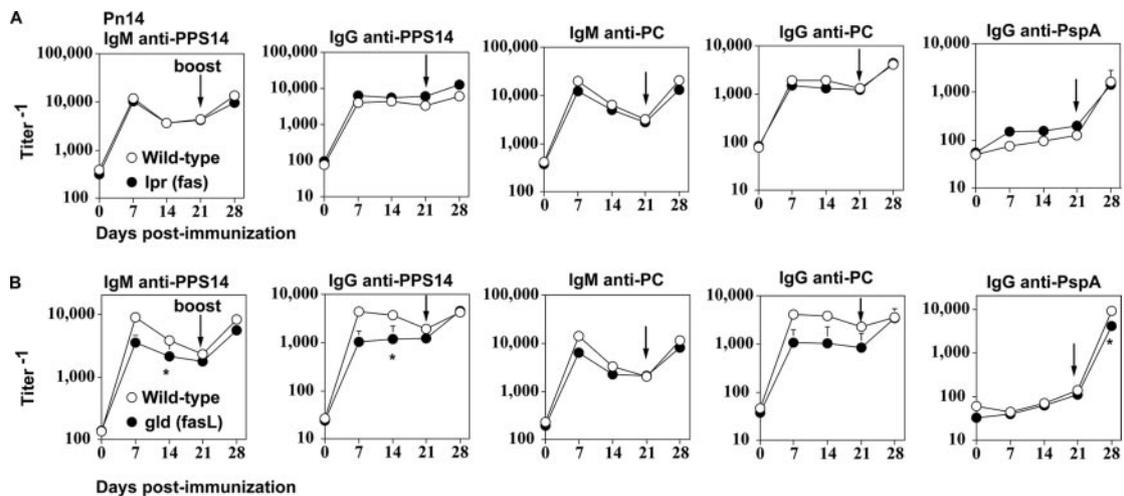
lpr (Fas-defective) and gld *(FasL-defective) mice do no elicit elevated anti-PS responses to intact Pn14*

The selective enhancement of anti-PS responses to intact Pn14 in mice constitutively expressing either Bcl-x$_L$ or Bcl-2 under the control of the Igh enhancer, suggested that B cell apoptosis in WT mice might limit these responses in vivo. Fas-mediated signaling in B cells is one potential, though nonexclusive, pathway that mediates B cell apoptosis (35). We thus wished to determine whether mice genetically defective in the Fas/FasL pathway might recapitulate the observations made in the Bcl-x$_L$-Tg or Bcl-2-Tg mice, in response to intact Pn14. We thus immunized *lpr* (Fas-defective) and *gld* (FasL-defective) mice and their WT controls (C3H/HeJ) i.p. with intact Pn14 in saline, followed by boosting on day 21. Neither *lpr* or *gld* mice exhibited elevations in primary or secondary IgM and/or IgG re-

sponses specific for PPS14, PC, or PspA (Fig. 3). A modest decrease in primary IgM and IgG anti-PPS14 responses was observed in *gld* relative to WT mice. These data suggest that a Fas/FasL-independent pathway of apoptosis likely plays a significant role in limiting anti-PS response to intact Pn14. Although C3H/HeJ mice, used as a control for *lpr* and *gld* mice, have a nonfunctional *TLR4* gene (36), they elicit Ig responses to Pn14 similar to their WT C3H/HeN controls (data not shown).

Bcl-x$_L$-Tg and Bcl-2-Tg, relative to WT, B cells exhibit more sustained DNA synthesis in vitro in response to multivalent mIg cross-linking, but not in response to CD40- or LPS-mediated signaling

We recently reported that anti-PS responses to intact Pn14 were more dependent than antiprotein responses, on Btk-dependent mIg

FIGURE 3. MRL/*lpr* (Fas-defective) and *gld* (FasL-defective) mice do no elicit elevated anti-PS responses to intact Pn14. *A*, *lpr* and WT control (C3H/HeJ; seven mice per group) and, *B*, *gld* and WT control (C3H/HeJ; seven mice per group) mice were immunized i.p. with 2 × 10^8 CFU equivalents of heat-killed Pn14 in saline. Mice were similarly boosted on day 21. Sera were collected on the indicated days for measurement of Ag-specific IgM and IgG isotype titers. Data are presented as geometric mean ± SEM; *, significance $p < 0.05$. The experiments illustrated in *A* and *B* were each performed once.

signaling (12). We thus wished to determine whether the enhancements in anti-PS responses in Bcl-x_L-Tg and Bcl-2-Tg mice in response to Pn14 might be explained, at least in part, by an increased responsiveness of Tg B to mIg cross-linking. We used dextran-conjugated anti-IgD Abs ($\alpha\delta$-dex), which we previously demonstrated to be an in vitro model of multivalent mIg cross-linking in response to PS Ags (37). Purified splenic B cells (B220$^+$) from WT or Tg mice were stimulated in vitro with $\alpha\delta$-dex and/or LPS or agonistic anti-CD40 mAb, each at a dose that induces optimal B cell proliferation. [^{3}H]Thymidine incorporation, as a measure of DNA synthesis, was determined on each of days 1–5 (Fig. 4A). Bcl-x_L-Tg and Bcl-2-Tg B cells exhibited substantially higher peak responses (day 3) than WT (day 2) B cells to $\alpha\delta$-dex stimulation (Bcl-x_L-Tg: 4.2-fold, Bcl-2-Tg: 4.6-fold), Additionally, Tg B cells maintained high levels of DNA synthesis, relative to the peak response, on days 4 and 5 (Bcl-x_L-Tg: 74 and 58% of peak, Bcl-2-Tg: 43 and 50% of peak, WT: 11 and 11% of peak, respectively). In contrast, WT and Tg B cells activated with either anti-CD40 mAb or LPS made comparable responses, except for 1.6- and 2.7-fold higher responses of LPS-activated Bcl-x_L-Tg and Bcl-2-Tg B cells, respectively, on day 5 (Fig. 4A).

As illustrated in Fig. 4A combined stimulation with a synergistic combination of $\alpha\delta$-dex plus LPS or $\alpha\delta$-dex plus anti-CD40 mAb resulted in roughly comparable peak responses (day 3) between WT and Tg B cells that was higher than that observed for any of the three stimulants alone. Of note, whereas responses of WT B cells declined significantly on days 4 and 5, Tg B cell responses continued to rise modestly. Additionally, early during the culture (days 1 and 2) the response of Tg B cells lagged behind that of WT B cells, with WT B cells showing higher levels of DNA synthesis. Intact Pn14 contains a number of distinct TLR ligands. In light of the synergy between $\alpha\delta$-dex and LPS, for DNA synthesis, we wished to determine whether $\alpha\delta$-dex and Pn14 also synergized for DNA synthesis and whether the response of Tg B cells to $\alpha\delta$-dex plus Pn14 differed from that of WT B cells. As illustrated in Fig. 4B, both WT and Tg B cells synthesized DNA only modestly to Pn14 alone. Similar to that observed in Fig. 4A, Tg B cells maintained sustained levels of DNA synthesis in response to $\alpha\delta$-dex alone (days 3–5), whereas the WT B cell response declined dramatically during this period. Of note, combined activation with $\alpha\delta$-dex plus Pn14 was synergistic for DNA synthesis by both WT and Tg B cells, with Tg B cells exhibiting significantly higher responses from days 2–5 relative to WT B cells (Fig. 4B).

Bcl-x_L-Tg and Bcl-2-Tg, relative to WT, B cells exhibit greater clonal expansion in response to $\alpha\delta$-dex
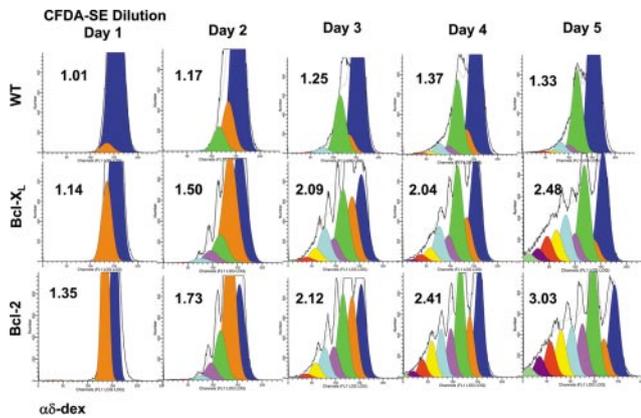
The selective enhancement in DNA synthesis of Bcl-x_L and Bcl-2-Tg B cells in response to $\alpha\delta$-dex activation, further suggested that Tg B cells also underwent a greater degree of clonal expansion than WT B cells. To determine this, we labeled purified splenic B cells (B220$^+$) from WT and Tg mice with CFDA-SE and measured the degree of CFDA-SE dilution, by flow cytometry, on various days following $\alpha\delta$-dex activation. A progressive 50% reduction in CFDA-SE fluorescence follows each round of proliferation. The overall clonal expansion of the B cell population can then be expressed as a PI (see Materials and Methods). As illustrated in Fig. 5, Bcl-x_L-Tg and Bcl-2-Tg B cells stimulated with $\alpha\delta$-dex demonstrated a higher PI on each of days 1–5 relative to WT B cells. Of note, whereas the PI of WT B cells progressively increased up to day 4 and then decreased slightly on day 5, the PI of Tg B cells continued to increase up to day 5. Thus, the more sustained levels of DNA synthesis of Tg, relative to WT, B cells in response to $\alpha\delta$-dex correlated with a greater degree of clonal expansion.

Bcl-x_L-Tg and Bcl-2-Tg B cells undergo less apoptosis than WT B cells in response to $\alpha\delta$-dex

In a final set of analyses, we determined whether the enhanced DNA synthesis and clonal expansion of Tg B cells in response to $\alpha\delta$-dex reflected a decrease in apoptosis relative to WT B cells. To determine this, we measured percentages of hypodiploid nuclei of WT and Tg B cells stimulated for 3–5 days with $\alpha\delta$-dex. As illustrated in Fig. 6, $\alpha\delta$-dex-activated WT B cells exhibited a reproducibly higher percentage of hypodiploid nuclei on days 3–5 relative to Tg B cells. By day 5, 81% of $\alpha\delta$-dex-activated B cells were apoptotic on the basis of hypodiploid nuclei, whereas only 37 and 26% of Bcl-x_L-Tg and Bcl-2-Tg B cells, respectively, were apoptotic Thus, transgenic expression of Bcl-x_L and Bcl-2 inhibited B cell apoptosis in response to $\alpha\delta$-dex throughout the latter period of culture, which likely contributed to the greater clonal expansion of individual $\alpha\delta$-dex-activated Tg B cells illustrated in Fig. 5.

The IgG anti-PPS14 response to Pn14 is markedly reduced in Lsc$^{-/-}$ mice

The increase in absolute numbers of splenic B-1 and peritoneal B-1b and B-2 cells in Tg mice, as well as the enhanced mitogenic response of Tg, relative to WT, B cells following mIg cross-linking suggest two possible, mutually inclusive, mechanisms for the

FIGURE 4. Bcl-x_L-Tg and Bcl-2-Tg, relative to WT, B cells exhibit more sustained DNA synthesis in vitro in response to multivalent mIg cross-linking, but not in response to CD40- or LPS-mediated signaling. A and B, Purified B220$^+$ splenic B cells were cultured at 2.5×10^5 cells/ml (3 wells/group) in the presence of the indicated stimuli (LPS = 10 μg/ml, anti-CD40 mAb = 10 μg/ml, $\alpha\delta$-dex = 10 ng/ml, and/or heat-killed Pn14 = 1×10^8 CFU/ml). [^{3}H]TdR was added to independent wells on days 1–5 and cells were harvested 18 h later for determination of DNA synthesis via incorporated cpm. Data are presented as geometric mean ± SEM; *, significance $p < 0.05$. One of two similar experiments is shown for both A and B.
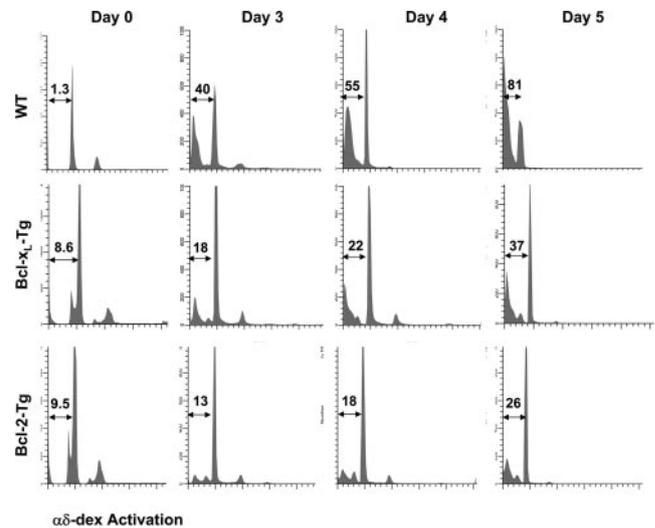
FIGURE 5. Bcl-x_L-Tg and Bcl-2-Tg, relative to WT, B cells exhibit greater clonal expansion in response to $\alpha\delta$-dex. CFDA-SE-loaded, purified B220$^+$ B cells from Bcl-x_L-Tg, Bcl-2-Tg, and WT (BALB/c) mice were cultured for varying times at 5×10^5 cells/ml in the presence of $\alpha\delta$-dex (10 ng/ml). Cells were analyzed by flow cytometry and PI was calculated (*upper left*). One of two similar experiments is shown.

FIGURE 6. Bcl-x_L-Tg and Bcl-2-Tg B cells undergo less apoptosis than WT B cells in response to $\alpha\delta$-dex. Purified B220$^+$ B cells from Bcl-x_L-Tg, Bcl-2-Tg, and WT (BALB/c) mice were cultured for 3–5 days at 5×10^5 cells/ml in the presence of $\alpha\delta$-dex (10 ng/ml). Cells were harvested and nuclei were stained with propidium iodide and analyzed by flow cytometry. The number above the horizontal arrow is the percentage of hypodiploid (apoptotic) nuclei. One of two similar experiments is shown.

enhanced anti-PS response to Pn14 in Tg mice. To better resolve the underlying mechanism(s), we first wished to determine the B cell subset(s) that give rise to the anti-PS response to Pn14. Mice genetically deficient in Lsc (Lsc$^{-/-}$) on the C57BL/6 background exhibit a marked defect in MZB migration from the marginal zone following immunization, precluding MZB interaction with CD4$^+$ T cells (25). Lsc acts selectively on MZB cells (25, 38). Thus, the TD IgM anti-NP response to NP-CG, which is dependent on MZB, is markedly reduced in Lsc$^{-/-}$ mice, whereas the TI anti-NP response to NP-Ficoll is not (25). The IgG anti-PPS14, anti-PC, as well as the anti-PspA, responses are dependent on CD4$^+$ T cells, whereas the IgM anti-PPS14 and anti-PC responses are TI (10, 39). We thus used the Lsc$^{-/-}$ mouse to determine whether any of these TD IgG responses were derived from MZB cells.

Lsc$^{-/-}$ mice were immunized i.p. with Pn14 and then boosted i.p. with Pn14, 14 days later. Sera were obtained on days 0, 7, 14, and 21 for determination of Ag-specific IgM and IgG titers. As illustrated in Fig. 7, *upper panel*, Lsc$^{-/-}$ mice exhibited a nearly complete abrogation in the primary and secondary IgG anti-PPS14 response. In contrast, the IgG anti-PC and anti-PspA responses were comparable between Lsc$^{-/-}$ and WT mice, as well as the IgM anti-PPS14 and anti-PC responses. Thus, these data strongly suggest that the IgG anti-PPS14 response to Pn14 is derived almost entirely from MZB cells. In distinct contrast to Pn14, Lsc$^{-/-}$ mice immunized with a mixture of two soluble pneumococcal conjugates (PPS14-PspA plus C-PS-PspA) in alum plus CpG-ODN, elicited primary and secondary IgG anti-PPS14 responses comparable to WT mice, in addition to similar IgM and IgG anti-PC, and IgG anti-PspA responses (Fig. 7, *lower panel*). These data further suggest that the IgG anti-PPS14 response to soluble conjugate vaccine, in contrast to intact Pn14, is derived from FB cells.

Both MZB and FB cells from Bcl-2-Tg, in contrast to WT, mice exhibit markedly higher levels of DNA synthesis late in culture, following mIg cross-linking

The observation that the IgG anti-PPS14 response to Pn14 appears to derive almost exclusively from MZB cells, and the roughly equivalent absolute numbers of MZB cells in Tg and WT mice, suggests that the prolongation and elevation of this response in Bcl-x_L-Tg and Bcl-2-Tg mice, might reflect a more sustained mitogenic response of Tg MZB cells following mIg cross-linking, as we demonstrated for unfractionated splenic B cells (Figs. 4 and 5).

To determine this, we purified splenic MZB and FB cells from Bcl-2-Tg and WT mice by electronic cell sorting and cultured them in the presence of $\alpha\delta$-dex, F(ab$'$)$_2$ anti-IgM, LPS, or agonistic anti-CD40 mAb, for 4 or 5 days, time points during which the mitogenic response of WT B cells are in substantial decline (see Fig. 4). As illustrated in Fig. 8A, WT MZB cells elicited a significantly higher LPS response than FB cells. In contrast, FB cells responded much more vigorously to mIg cross-linking than MZB cells (Fig. 8), whereas responses to anti-CD40 mAb were comparable. These responses are consistent with previous reports (40, 41). Of note, DNA synthesis in Bcl-2-Tg MZB cells in response to mIg cross-linking ($\alpha\delta$-dex or F(ab$'$)$_2$ anti-IgM) was 20- to 95-fold higher than WT MZB cells on day 4 or 5 (Fig. 8). A smaller, but significant, enhancement in DNA synthesis (4.4-fold) was observed in Tg, relative to WT, B cells activated with anti-CD40 mAb (Fig. 8B), whereas LPS responses were comparable (Fig. 8A). DNA synthesis in Tg FB cells activated via mIg cross-linking was also dramatically enhanced (13- to 68-fold) on day 4 or 5, relative to WT FB cells, roughly comparable to the fold increases observed in Tg vs WT MZB cells (Fig. 8). A smaller, but significant, enhancement in the LPS response of Tg relative to WT FB cells (3.6 and 5.1) on days 4 and 5, respectively was also observed (Fig. 8A), whereas no significant differences were observed in response to anti-CD40 mAb (Fig. 8B). Collectively, these data support the notion that, at least for the IgG anti-PPS response to Pn14, the more prolonged and elevated responses in Bcl-x_L-Tg and Bcl-2-Tg mice reflect a more sustained mitogenic response of PPS14-specific B cells following immunization, likely due to decreased apoptosis.

Discussion

This study makes the following major, novel observations: 1) B cell-specific transgenic expression of either Bcl-x_L or Bcl-2 results in a selective enhancement of the primary IgM and IgG anti-PS, in contrast to the antiprotein, response to intact Pn14; this enhancement in the anti-PS response is not observed in response to isolated PS Ags or to PS-protein conjugate, emphasizing the unique nature
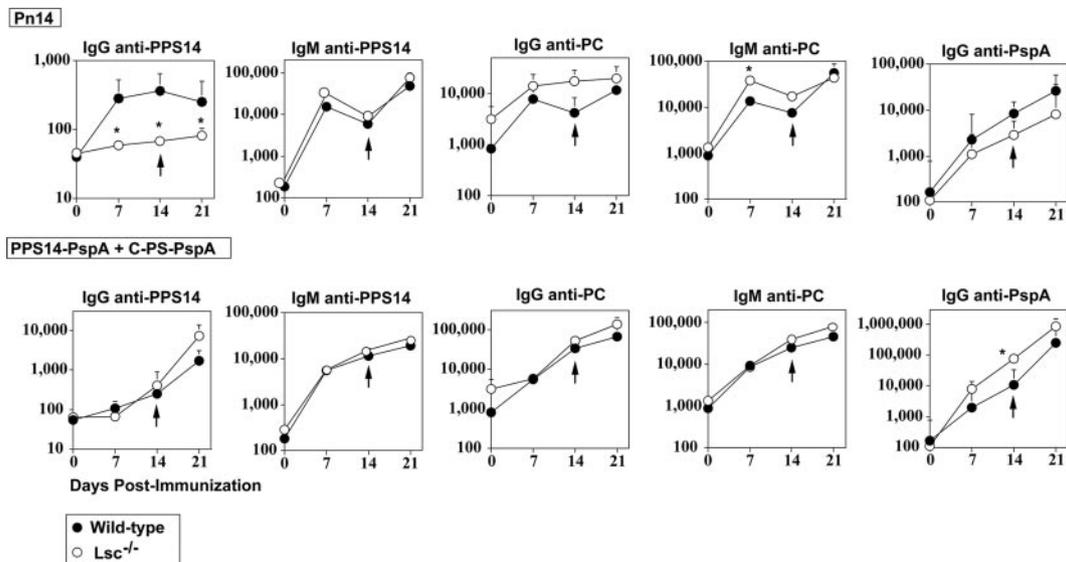
FIGURE 7. The IgG anti-PPS14 response to Pn14 is markedly reduced in Lsc$^{-/-}$ mice. Lsc$^{-/-}$ and WT mice (seven per group) were immunized i.p. with either 2×10^8 CFU equivalents of heat-killed Pn14 in saline (*upper panel*) or i.p. with 1 μg each of PPS14-PspA and C-PS-PspA suspended in alum + CpG-ODN (*lower panel*). Mice were similarly boosted with their respective immunogens on day 14. Sera were collected on the indicated days for measurement of Ag-specific IgM and IgG isotype titers. Data are presented as geometric mean ± SEM; *, significance $p < 0.05$. One of two similar experiments is shown.

of the intact bacterium as an immunogen. 2) Despite a more prolonged and higher anti-PS response to Pn14 in Tg mice, PS-specific memory is still not generated. 3) Genetic disruption of Fas/FasL signaling has no enhancing effect on either the anti-PS or antiprotein response to Pn14, strongly suggesting that transgenic Bcl-x$_L$ and Bcl-2 enhance anti-PS responses in a Fas-independent manner. 4) Bcl-x$_L$-Tg and Bcl-2-Tg mice exhibit higher absolute numbers of splenic B-1 and peritoneal B-1b and B-2 cells relative to WT mice, cells known or suspected to play a selective role in anti-PS responses. Notably, however, Tg and WT mice have comparable absolute numbers of MZB cells, a subset also strongly implicated in PS-specific immunity. 5) Unfractionated Tg B cells undergo more sustained DNA synthesis in response to multivalent mIg cross-linking, but not in response to agonistic anti-CD40 mAb or LPS, associated with greater clonal expansion of Tg B cells and lower levels of apoptosis. 6) Studies in the Lsc$^{-/-}$ mouse strongly suggest a critical role for the MZB, at least for the IgG anti-PPS14 response to intact Pn14, but not soluble pneumococcal conjugate, the latter response likely arising from FB cells. 7) The marked enhancement in DNA synthesis in Tg MZB cells following mIg cross-linking suggests one potential mechanism for the enhanced Pn14-induced IgG anti-PPS14 response.

PS Ags, unlike proteins, contain repeating identical antigenic epitopes capable of multivalent mIg cross-linking (1). Dextran-conjugated anti-IgD mAbs ($\alpha\delta$-dex), an in vitro polyclonal model for PS-mediated multivalent mIg cross-linking (42), has been shown to deliver potent mitogenic signals to B cells at doses of anti-Ig that are 1000-fold lower than that observed using unconjugated, bivalent anti-Ig Ab (27). Additionally, in response to the same set of costimulating cytokines, $\alpha\delta$-dex often induces a different functional outcome, relative to LPS or CD40-mediated activation (43). These data suggest that mIg signaling in response to PS Ags may be quantitatively and qualitatively distinct from that which occurs upon contact with a protein Ag. In this regard, we recently demonstrated that mice with reduced, though not absent, Btk-mediated mIg signaling in B cells, while having largely restored B cell development (44, 45) elicit significantly lower IgM and IgG anti-PS, but not IgG antiprotein, responses to intact Pn14

(12). Of note, MZB cells, which are known to play a role in anti-PS responses, including Pn (46, 47), and directly implicated, in this study, in the IgG anti-PPS14 response to Pn14, proliferate relatively poorly in vitro upon multivalent mIg cross-linking alone (40), relative to FB cells, despite exhibiting higher levels of proliferation upon activation with LPS (40, 41). Our demonstration that Bcl-x$_L$-Tg and Bcl-2-Tg B cells activated with $\alpha\delta$-dex exhibit a more prolonged, high-level of DNA synthesis, associated with enhanced clonal expansion, and decreased apoptosis, relative to WT B cells, suggests one potential mechanism for the selective prolongation and higher peak levels of the primary in vivo anti-PS response in Tg mice. Our further observation of markedly elevated levels of DNA synthesis in Tg MZB, as well as FB, cells following mIg cross-linking, combined with the comparable numbers of MZB cells in unimmunized Tg and WT mice, lend strong support to this notion. This mechanism, and/or the increased numbers of splenic B-1, and/or peritoneal B-1b and B-2 cells in Tg mice could additionally account for the higher IgM anti-PPS14, and IgM and IgG anti-PC, responses also observed in Tg mice.

In contrast to our data, an earlier study failed to demonstrate significant differences in DNA synthesis between Bcl-2-Tg and WT splenic B cells 4 days after in vitro activation with $\alpha\delta$-dex (48). However, this latter study used a different Bcl-2-Tg mouse model, established by Korsmeyer and colleagues (49), which lacked insertion of the Eμ enhancer 5' of the *bcl-2* transgene, and additionally was backcrossed onto the CBA/CaJ mouse background. We used Bcl-2-Tg mice established by Strasser et al. (50) which contained an Eμ enhancer 5' to the *bcl-2* transgene and was extensively backcrossed onto the BALB/c mouse background. One potential difference between the two models could be the relative expression of Bcl-2 protein in the B cells, affecting the functional response to $\alpha\delta$-dex-mediated mIg cross-linking.

A previous report demonstrating that Bcl-2-Tg B cells have an enhanced Ca^{2+} influx relative to WT B cells in response to mIg cross-linking (51), is consistent with the altered functional responsiveness of Tg B cells to this general mode of activation. Although forced expression of Bcl-x$_L$ and Bcl-2 has been shown to delay cell cycle entry (52, 53), the initial kinetics of DNA synthesis and
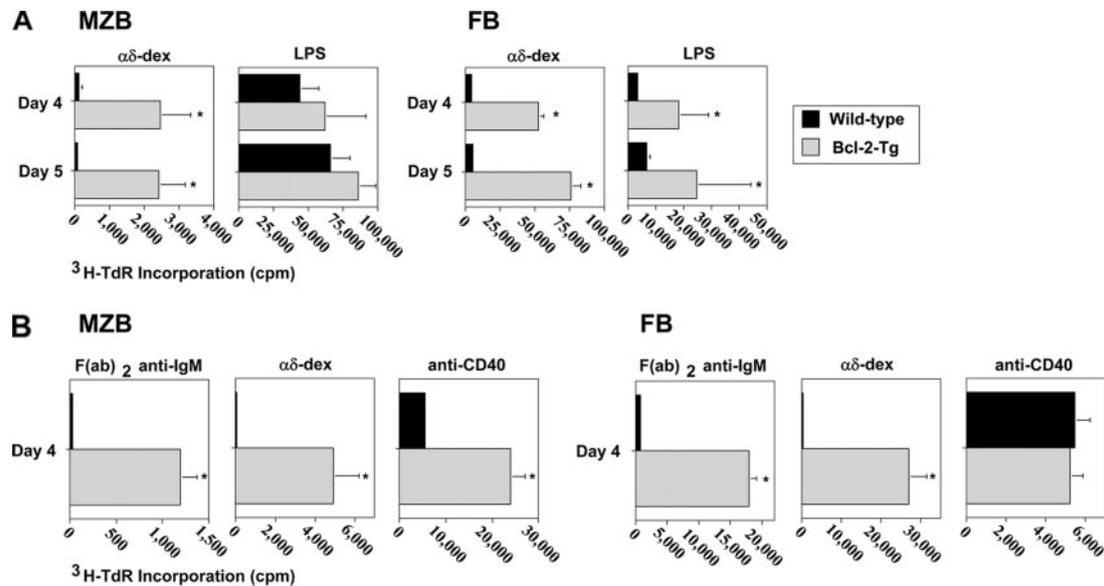
FIGURE 8. Both MZB and FB cells from Bcl-2-Tg, in contrast to WT, mice exhibit markedly higher levels of DNA synthesis late in culture, following mIg cross-linking. Purified MZB or FB cells were cultured at 2.5×10^5 cells/ml (3 wells/group) in the presence of the indicated stimuli (LPS = 10 μg/ml, anti-CD40 mAb = 10 μg/ml, $\alpha\delta$-dex = 10 ng/ml, or F(ab')$_2$ goat anti-mouse IgM (10 μg/ml)). [^{3}H]TdR was added to independent wells on days 4 and/or 5 and cells were harvested 18 h later for determination of DNA synthesis via incorporated cpm. Data are presented as geometric mean ± SEM; *, significance $p < 0.05$.

proliferation of WT and Tg B cells activated with $\alpha\delta$-dex alone are similar. However, some delay in initial DNA synthesis is apparent in Bcl-x$_L$-Tg and Bcl-2-Tg B cells activated with $\alpha\delta$-dex plus LPS or $\alpha\delta$-dex plus anti-CD40, and Bcl-2-Tg B cells activated with $\alpha\delta$-dex plus Pn14. Although MZB cells express substantially lower levels of mIgD than FB cells, which could potentially have a differential impact on their response to $\alpha\delta$-dex, similar observations were also made using F(ab')$_2$ anti-IgM Ab.

The likelihood that PS-specific B cells are more prone than protein-specific B cells to undergo apoptosis in response to Pn14 in vivo may in part reflect the relatively shorter period during which they receive CD4$^+$ T cell help, presumably including CD40-mediated activation, for the IgG anti-PS response, and the T cell-independence of the IgM anti-PS response (9–11). Additionally, MZB cells exhibit increased apoptosis in response to mIg cross-linking alone, relative to FB cells (41). Of note, CD40-mediated activation can prevent both MZB and FB cells (41), as well as immature and GC B cells (54–56) from undergoing mIg-induced apoptosis. Thus, the more prolonged period of CD4$^+$ T cell help for the antiprotein response to Pn14 may confer increased protection against apoptotic cell death and allow for a more prolonged humoral immune response to occur. The typical provision of a relatively higher level and/or duration of promitogenic stimulation for WT FB cells may account for the similar IgG anti-PspA responses to Pn14, as well as the similar IgG anti-PPS14 and IgG anti-PspA responses to pneumococcal conjugate in WT and Tg mice, despite a higher in vitro proliferative response of Tg FB cells following mIg cross-linking.

Our studies in the Lsc$^{-/-}$ mouse directly implicate MZB cells in the IgG anti-PPS14 response to Pn14, but not to soluble pneumococcal conjugate. In the absence of the intracellular protein, Lsc, MZB cells do not detach efficiently from integrin ligands within the marginal zone, which likely limits their entry into the T cell zone for receipt of T cell help (25, 57). Indeed, the TD IgM anti-NP response to NP-CG, which is dependent on MZB, is markedly reduced in Lsc$^{-/-}$ mice, whereas the TI anti-NP response to NP-Ficoll is not (25). Lsc deficiency affects MZB cells to a much

greater extent than FB cells, likely due to relatively increased levels of Lsc protein expressed by MZB cells (38). The Lsc$^{-/-}$ mouse strain used in this study exhibited similar proportions of MZB and FB cells in the spleen relative to WT mice, although absolute numbers were decreased 50% (25). In contrast, no significant differences in the numbers of splenic T cells were observed. Although Lsc$^{-/-}$ mice were reported to have an 80% decrease in peritoneal B-1 cells (25), our observations of normal IgM and IgG anti-PC, and IgM anti-PPS14 responses in Lsc$^{-/-}$ mice, strongly argue that this reduction did not significantly impact on the overall anti-Pn14 Ig response. B-1 B cells have previously been implicated in anti-PC responses, in particular the TI response to Pn (47). It is also highly unlikely that the abrogation of the TD IgG anti-PPS14 response in Lsc$^{-/-}$ mice was instead, due to defective CD4$^+$ T cell help. First, Rubtsov et al. (25) demonstrated a normal TD IgG response to their two TD Ags in Lsc$^{-/-}$ mice. Second, our data demonstrate a normal IgG anti-PC and IgG anti-PspA response to intact Pn14 and normal IgG anti-PPS14, PC, and PspA responses to soluble conjugate vaccine in Lsc$^{-/-}$ mice, responses that are all CD4$^+$ TD (10, 58).

We believe that our use of Lsc$^{-/-}$ mice to establish a role for MZB cells in the IgG anti-PPS14 response was more compelling than the traditional approach of adoptive transfer of B and T cells into lymphopenic mice. As reviewed by Singh and Schwartz (59) this latter approach has a number of potential pitfalls. These include homeostatic proliferation, changes in phenotype, changes in function, and alterations in cellular trafficking of the donor cells. Indeed, we observed that the majority of sorted-purified splenic B220$^+$CD23$^+$CD21^{intermed} cells (FB) transferred into RAG$^{-/-}$ mice had lost expression of CD23 and CD21, but not B220, 2 days following transfer (data not shown). A similar loss of CD21, but not B220, from a majority of B220+CD23$^{-/-}$CD21^{high} cells (MZB) was also observed. Thus, the potential advantage of the Lsc$^{-/-}$ mouse over adoptive transfer into a lymphopenic mouse is that the lymphoid architecture in the Lsc$^{-/-}$ mouse is intact before immunization, and there is no ex vivo manipulation of lymphocyte

cell subsets or effects of transferring cells into a lymphopenic environment. A report by Haas et al. (32) using the adoptive transfer approach, demonstrated that the majority of the anti-PPS3 IgM and IgG3 response to purified soluble PPS3 is not mediated by splenic MZ B cells but by peritoneal B1b cells. These data do not necessarily conflict with our own, because the immunogens used in these respective studies were significantly different. Thus, the IgG anti-PPS response to soluble PPS is TI, whereas the IgG anti-PPS response to intact Pn is CD4$^+$ TD. The former Ag is soluble, whereas our immunogen is particulate. Indeed, a major role for splenic MZB, as well as B-1, cells in the IgM anti-PC response to intact Pn has been demonstrated (47).

The equivalent anti-PS responses to pneumococcal conjugate vaccine in WT and Lsc$^{-/-}$ mice, in addition to WT and Bcl-x$_L$-Tg mice, further suggest that this response is mediated by FB cells, as opposed to the involvement of MZB cells in the response to intact Pn14. In this regard, pneumococcal conjugate, but not intact Pn14, elicits memory for the IgG anti-PPS14 response (12). Although MZB cells are potent activators of naive CD4$^+$ T cells, MZB cells appear to be less prone to developing into memory cells than FB cells (57, 60). Our further demonstration that WT and Bcl-x$_L$-Tg mice elicit equivalent Ig responses to isolated PS Ags, in contrast to Pn14, is consistent with a previous study using NP-Ficoll in Bcl-2-Tg mice (61). In this regard, it is possible that Tg expression of Bcl-x$_L$ or Bcl-2 may not compensate for the absence of classical T cell help for Ig responses to purified, soluble PS Ags.

Our observation of normal primary and secondary antiprotein Ig responses in Bcl-x$_L$-Tg mice in response to either intact Pn14 or a soluble pneumococcal conjugate is largely consistent with a previous study by Takahashi et al. (62). Thus, they reported that in response to i.p. injection of NP-CGG in alum, mice transgenic for Bcl-x$_L$ in the B cell compartment briefly expressed higher numbers of splenic NP-specific Ab-forming cells (AFCs) after immunization but did not increase the number or size of GCs, alter the levels of serum Ab, or change the frequency of long-lived AFCs. However, an increase in low affinity NP-specific B cell clones in the spleen, decreased average affinity of both long-lived NP-specific AFCs in the BM, and NP-specific serum Ig was observed in the Tg mice. Similarly, Bcl-2-Tg mice immunized i.p. with TNP-OVA in Alu-Gel-S elicited an anti-TNP Ig response, affinity maturation, and GC formation similar to WT mice (51). In contrast, it has been demonstrated that Bcl-2-Tg mice immunized i.p. with NP-KLH in alum exhibited an increase in the frequency of AFCs in the spleen and bone marrow, and no decrease in affinity of bone marrow AFCs or serum Ig (63, 64). Similarly, Bcl-2-Tg mice immunized with NP-CGG in alum elicited higher serum titers of IgM and IgG anti-NP, relative to WT mice (65). Bcl-2-Tg mice immunized with SRBC also produced an amplified and protracted Ab response (50). The basis for the conflicting results using isolated protein Ags are unclear, especially given that Bcl-x$_L$ and Bcl-2 proteins appear to be equipotent in promoting B cell survival and may act through similar mechanisms (13).

We make the novel observation that the absolute numbers of both splenic B-1 and peritoneal B-1b cells are elevated in both Bcl-x$_L$-Tg and Bcl-2-Tg mice. Both of these B cell subsets have been implicated in PS-specific and/or T cell-independent Ig responses (30–33). Bcl-x$_L$-Tg and Bcl-2-Tg mice also exhibit an increase in peritoneal B-2 cells, a B cell subset considered intermediate in function between peritoneal B-1 and splenic B-2 cells, with some capacity to secrete natural Ig (34). Bcl-2-Tg mice were earlier shown to exhibit an overall increase in splenic B220$^+$ cells and a selective decrease in the percentage, but not the absolute number, of CD21^{high}CD23^{low} B cells (MZB phenotype), relative to WT mice (49, 61, 65, 66). In addition,

similar to our data, peritoneal B-2 cells were previously shown to be elevated in Bcl-2-Tg mice, whereas total peritoneal B-1a cells were present in normal numbers (61). The numbers of peritoneal B-1b and splenic B-1 cells, found in our study to be elevated in both Bcl-x$_L$-Tg and Bcl-2-Tg mice, were not reported in this latter study. Bcl-x$_L$ Tg mice were also previously shown to exhibit an increase in B220$^+$IgM$^+$ spleen cells, relative to WT mice (62). Collectively, these data suggest that the higher absolute numbers of particular B cell subsets that have been previously implicated in anti-PS responses could contribute at least in part, to the enhanced peak primary anti-PS responses to Pn14 in Tg mice.

Engagement of cell surface Fas/APO-1 (CD95) on B cells can induce B cell apoptosis (35). Whereas, naive B cells express only low levels of Fas, activation via CD40 or LPS, although not mIg cross-linking, substantially up-regulate Fas and subsequent sensitivity to apoptotic cell death. Of interest, concomitant signaling of CD40-activated B cells via mIg significantly decreases Fas-sensitivity relative to B cells activated via CD40 alone, without decreasing Fas expression itself (67–69). Resistance to Fas-mediated B cell apoptosis can also be mediated by IL-4 (70), engagement of MHC class II (71), and TLR9-mediated signaling (72). Thus, B cells exhibit numerous mechanisms for resisting Fas-mediated apoptosis, although germinal center B cells express relatively high levels of Fas (18, 63) and are susceptible to Fas-mediated apoptosis in vitro (73, 74). In this regard, the anti-PS response to intact Pn14 is dependent on cognate CD4$^+$ T cell help, CD40- and mIg-mediated activation, TLR-dependent signaling, and is regulated by endogenous IL-4 (9, 10, 12, 58, 75), factors that could combine to confer Fas resistance. The ability of Bcl-x$_L$ or Bcl-2 to inhibit Fas-mediated B cell apoptosis is still a matter of controversy based on conflicting reports (35). The *lpr* and *gld* genes encode defective forms of Fas (76) and FasL (77), respectively. In this regard, we show that neither *lpr* nor *gld* mice exhibit significant increases in either the primary or secondary anti-PS or antiprotein response to Pn14, strongly suggesting that the ability of transgenic Bcl-x$_L$ and Bcl-2 to enhance anti-PS responses was not via antagonism of Fas signaling. These data are consistent with a previous report demonstrating a normal anti-NP Ig response to i.p. immunization with NP-KLH in alum in *lpr* mice (63).

In summary, these data are the first to demonstrate that PS-specific B cells responding to an intact bacterium are likely to be more apoptosis prone, as evidenced by the observation that Bcl-x$_L$ and Bcl-2 can act in a similar fashion to selectively enhance anti-PS, relative to antiprotein, responses to Pn14, when expressed constitutively in a B cell-specific manner. The mechanism for this enhancement in the PS-specific Ig response appears to be Fas independent, and may reflect a combination of increased clonal expansion of PS-specific Tg B cells in response to PS-mediated multivalent mIg cross-linking and perhaps, higher absolute numbers of B cell subsets implicated in the anti-PS response.

Acknowledgments

Disclosures

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