Vol. 2 No. 13 BC
www.signaling-gateway.org/reports/v2/BC0013

Cultured Splenic B Cells Expressing a Human Bcl-2 Transgene as an Alternative Signaling Model for Primary B Cells

Tamara Roach, Sangdun Choi, Heping Han, and Xiaocui Zhu
Alliance for Cellular Signaling Laboratories
San Francisco Veteran's Administration Medical Center, San Francisco, CA; California Institute of Technology, Pasadena, CA; and University of Texas Southwestern Medical Center, Dallas, TX

Abstract: The Alliance for Cellular Signaling (AfCS) has sought to identify a suitable model cell type that can be used to study intracellular signaling networks in a detailed manner. Our laboratory examined cultured mouse splenic B lymphocytes (B cells) expressing a transgenic human bcl-2 gene (hbcl-2) and found them to be a promising model for long-term culture studies of primary B cells. The transgenic hbcl-2 B cells survived and maintained stable signaling responses for at least one week in culture, compared with wild-type C57BL/6 splenic B cells, which rapidly died. Signaling parameters measured in response to cell stimulation included intracellular calcium flux, phosphorylation of a panel of signaling proteins, chemotaxis, and changes in gene expression. Transgenic hbcl-2 B cells showed similar responses to freshly isolated wild-type B cells across these parameters. Wild-type B cell survival is notably less than that of transgenic hbcl-2 B cells, even when stimulated in culture with the prosurvival factors BAFF or anti-CD40 monoclonal antibody. Further, hbcl-2 transgenic B cells remain in a relatively quiescent state, unlike cells stimulated with BAFF or anti-CD40, which are activated. An unfortunate disadvantage of wild-type B cells that was also exhibited by the transgenic hbcl-2 B cells was their resistance to transfection or transduction. We attempted to use plasmid-based lentiviral systems that are capable of infecting many primary cells with high efficiency, but the B cells proved resistant to current methodologies. Although this trait is specifically forbidding for AfCS goals, it does not disqualify the hbcl-2 transgenic B cells from providing a stable, long-term culture system for study of splenic B cells.

Introduction

Splenic B lymphocytes (B cells) from C57BL/6 (B6) mice were one of the original models for cell signaling studies used by the Alliance for Cellular Signaling (AfCS). While freshly isolated wild-type B6 splenic B cells are a suitable model for studying proximal membrane signaling, they rapidly die when cultured in the absence of proliferative and/or survival stimuli. Therefore, in cultured B cells, long-term signaling responses are measured against a background of cell death. The rapid death of cultured B cells also precludes using RNA interference (RNAi) or antisense oligodeoxynucleotides to manipulate signaling protein expression (1-4).

The AfCS therefore sought to define conditions that allow the sustained survival of B cells in culture and that might ultimately permit transfection or transduction of these cells for the manipulation of cell signaling. Specifically, we compared the survival in culture of fresh wild-type B cells with the survival of transgenic B cells expressing the human bcl-2 gene (hbcl-2). For the studies presented here, we used Eμ-bcl-2-22 mice, created by Suzanne Cory and her colleagues, in which hbcl-2 is expressed under the control of the Eμ enhancer (5). Overexpression of the human bcl-2 gene in mouse B cells prolongs their survival both in vivo and in vitro in the absence of stimulation (5-9). We compared the survival and functional properties of these hbcl-2 transgenic B cells with those of wild-type B cells grown in BAFF (also known as BlyS/TALL-1/zTNF-4) or with anti-CD40 antibody (a substitute for CD40 ligand). BAFF and anti-CD40 are two members of the tumor necrosis factor (TNF) superfamily that stimulate receptors on B cells known to enhance B cell survival (10-13).

This report describes the survival, phenotype, and signaling responses of cultured hbcl-2 transgenic B cells, compared with freshly isolated or stimulated (BAFF or anti-CD40) cultured wild-type B cells. We found that hbcl-2 transgenic B cell survival was approximately 80% after six days in culture (with serum), significantly greater than wild-type B cells, whether unstimulated or stimulated with either BAFF or anti-CD40. In addition, cultured hbcl-2 transgenic B cells retained a characteristic mature B cell surface phenotype, as determined by expression of IgM, B220, CD23, CXCR4, and CXCR5. In hbcl-2 transgenic B cells cultured for six days, signaling through the B cell receptor (BCR) was intact (as assessed by calcium transients, signaling protein phosphorylation, and upregulation of the activation markers CD86 and CD69) and was comparable to that of fresh B cells. Chemotactic responses to SDF-1α or BLC by cultured hbcl-2 transgenic B cells were also similar to those of fresh B cells, confirming the expression of functional chemokine receptors CXCR4 and CXCR5. The mRNA expression profiles for hbcl-2 transgenic B cells produced by DNA microarray analyses were relatively stable over time in culture and revealed a reproducible response to stimulation of the BCR even after three or six days. B cell proliferation in response to BCR ligation, CD40 ligation, or LPS stimulation revealed that both hbcl-2 transgenic and wild-type B cells were stimulated to synthesize DNA, as assessed by ^{3}H-thymidine incorporation, albeit hbcl-2 transgenic B cells showed lower levels of incorporation and reduced increases in cell numbers when counted at two days poststimulation. Disappointingly, cultured hbcl-2 transgenic B cells were relatively refractive to transfection or transduction, including lentiviral-mediated

transduction. Lentivirus vector systems have recently been used to implement gene silencing mediated by RNA interference (RNAi). Lentivirus transduction of sequences driving the transcription of small hairpin RNAs (shRNAs) processed into small interfering RNA (siRNA) duplexes has been used to functionally silence genes in activated T cells (14, 15). The inability to efficiently transduce or transfect B cells currently precludes the use of vector-based RNAi or antisense to manipulate signaling protein expression in these cells. Despite this shortcoming, we conclude that cultured hbcl-2 transgenic B cells provide a good model system to study primary B cell signaling responses due to their prolonged survival in culture and their similar surface phenotype and signaling responses to wild-type B cells.

Results and Discussion

Yield of hBcl-2 Transgenic B Cells

Transgenic mice expressing the human bcl-2 gene have enlarged spleens and demonstrate a polyclonal increase in the number of B cells in the spleen, lymph nodes, and bone marrow (5-7). We found that hbcl-2 transgenic mouse spleens (146 ± 19 mg, n = 14) were, on average, twice as large as spleens from wild-type littermate mice (76 ± 11 mg, n = 7) and contained four times as many B cells (Table 1). hBcl-2 transgenic B cells could be enriched to >95% by depletion of cells expressing CD3 (T cells), CD11b (myeloid cells, i.e., macrophages and neutrophils), and CD43 (expressed on most leukocytes, but not resting mature B cells) using the Miltenyi AutoMACS magnetic cell sorter (AfCS Procedure PP0000000100). However, this method nonspecifically removed some B cells, possibly due to B cells adhering to T cells or myeloid cells, or by B cell Fc receptor (FcR) interactions with antibody-coupled magnetic beads. Since the cell yields we obtained were sufficient (60%), and we wished to compare wild-type and bcl-2 transgenic B cells prepared with the same procedure, we did not further optimize the isolation protocol specifically for hbcl-2 transgenic B cells, other than to take total cell numbers per spleen into account for calculating the amounts of beads required for labeling, and for the processing of the bead-labeled cells.

Table 1. ***B cell yields from hbcl-2 transgenic and wild-type mouse spleens (6- to 8-week-old mice).***

		hBcl-2 Transgenic (n = 19)	Wild-type (n = 16)
Presort	Viable (%)	85.0%	83.7%
	Cells/spleen	$3.40 \pm 0.73 \times 10^8$	$1.10 \pm 0.05 \times 10^8$
	$B220^{pos}$ (%)	73.7%	55.0%
	$B220^{pos}$ Cells/spleen	2.5×10^8	0.60×10^8
Postsort Flow-Through (B Lymphocytes)	Viable (%)	94.4%	97.1%
	Cells/spleen	$1.57 \pm 0.39 \times 10^8$	$0.43 \pm 0.03 \times 10^8$
	$B220^{pos}$ (%)	**96.7%**	**95.8%**
	$B220^{pos}$ Cells/spleen	**1.51×10^8**	**0.41×10^8**
	Yield (%)	60.4%	68.3%
Postsort Eluted (CD43+, CD3+, or CD11b+)	Viable (%)	86.5%	90.6%
	Cells/spleen	$0.68 \pm 0.15 \times 10^8$	$0.36 \pm 0.02 \times 10^8$
	$B220^{pos}$ (%)	40.4%	24.5%
	$B220^{pos}$ Cells/spleen	0.27×10^8	0.09×10^8

Survival of hBcl-2 Transgenic B Cells in Culture

Wild-type B cells rapidly die in culture. Hence, we sought to define specific conditions that would allow the sustained survival of B cells for signaling studies. We compared several alternative potential primary cell signaling models, specifically hbcl-2 transgenic B cells and wild-type B cells stimulated with BAFF or anti-CD40 survival promoting ligands. Initial experiments using defined serum-free medium showed that after three days in culture, the survival of hbcl-2 transgenic B cells was 80% of the initial cells plated (Fig. 1). In contrast, the survival of wild-type B cells was less than 10%. Stimulation of wild-type B cells with BAFF or anti-CD40 increased viability to 37% and 43%, respectively (Fig. 1). From these results, we determined that the survival of hbcl-2 transgenic B cells was superior to that of either BAFF- or anti-CD40–stimulated wild-type B cells. Moreover, BAFF or anti-CD40–stimulated wild-type B cells were partly activated and more proliferative (data not shown), and yet the percentage of viable cell numbers remained relatively low (only 37% to 43%), such that assays performed with these populations contained significant numbers of dead or dying cells as well as cell debris. Therefore, wild-type B cells stimulated with BAFF and anti-CD40 were not investigated further as potential cultured cell models for studying primary cell signaling.

We then extended the period of culture of hbcl-2 transgenic B cells, comparing survival in serum-free medium to survival in medium supplemented with 10% fetal

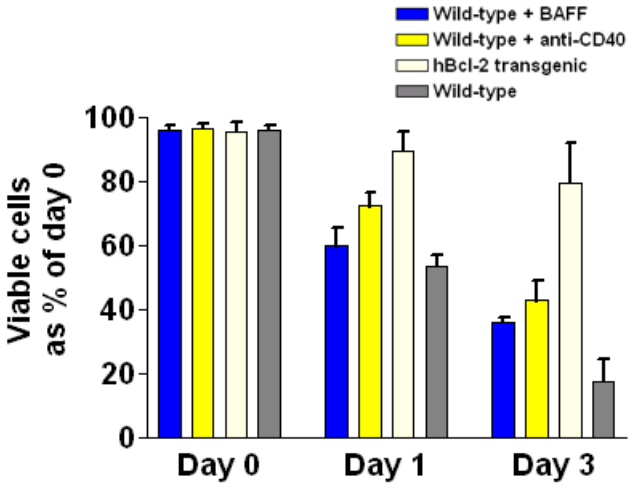

Fig. 1. **Cell viability during culture of hbcl-2 transgenic and wild-type B cells.** Splenic B cells were cultured in 6- or 24-well Costar ultralow plates at a density of 6×10^6/ml, with 3 ml or 0.6 ml/well, respectively, in serum-free medium (SIMDM). Wild-type B cells were stimulated with BAFF (3.7 nM = 62.5 ng/ml) or hamster IgM anti-CD40 (4.2 nM = 0.63 µg/ml) as indicated (anti-CD40 isotype control antibody had no significant effect on cell survival, data not shown). After one or three days in culture, cells were harvested, and viable cells were counted using trypan blue exclusion. The average viability data (±SD) for four experiments for each strain or treatment group is shown.

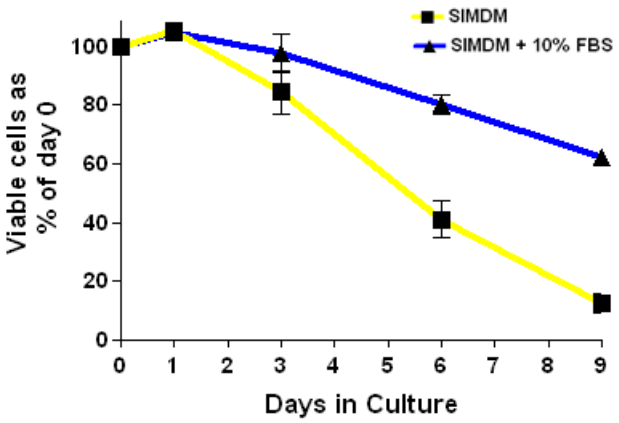

Fig. 2. **Human bcl-2 transgenic B cell viability in culture -/+10% FBS.** Human bcl-2 transgenic B cells were cultured at 6 x 10^6/ml in 24-well plates, using 0.6 ml/well SIMDM with or without 10% FBS added. On days 3 and 6, half of the culture medium was removed and replaced with fresh medium. On days 1, 3, 6, and 9, wells were harvested, and viable cells were counted after testing for trypan blue exclusion. The graphs show standard deviations for points where n = 3 experiments (i.e., three separate cultures of hbcl-2 transgenic B-cell preparations); for other points n = 2.

bovineserum (FBS). FBS improved survival: the survival of hbcl-2 transgenic B cells after six days of culture without serum was approximately 40%, but the survival with 10% FBS was approximately 80% (Fig. 2).

Surface Phenotype of hBcl-2 Transgenic B Cells

We compared the expression of surface antigens on hbcl-2 transgenic B cells to expression on wild-type mature splenic B cells. In accordance with earlier reports by Strasser and colleagues (5, 7), we observed that the expression levels of IgM, IgD, CD23 and B220 on splenic hbcl-2 transgenic B cells were comparable to levels observed on wild-type splenic B cells (Fig. 3). Therefore, the majority of splenic hbcl-2 transgenic B cells were mature B2 B cells (IgM^+, IgD^{+++}, $CD23^{++}$ and $B220^{+++}$; Fig. 3A and 3C, Hardy and Hayakawa,16), similar to wild-type splenic B cells (Fig. 3B and 3D). Marginal zone (MZ) B cells ($CD21^{+++}$, $CD23^-$; region R1, Fig. 4) were reduced in hbcl-2 transgenic mice. Marginal zone B cells comprised approximately 5% of the wild-type splenic B cell population but were barely detected in hbcl-2 transgenic splenic B cells (R1 = <1%). Follicular, mature B2

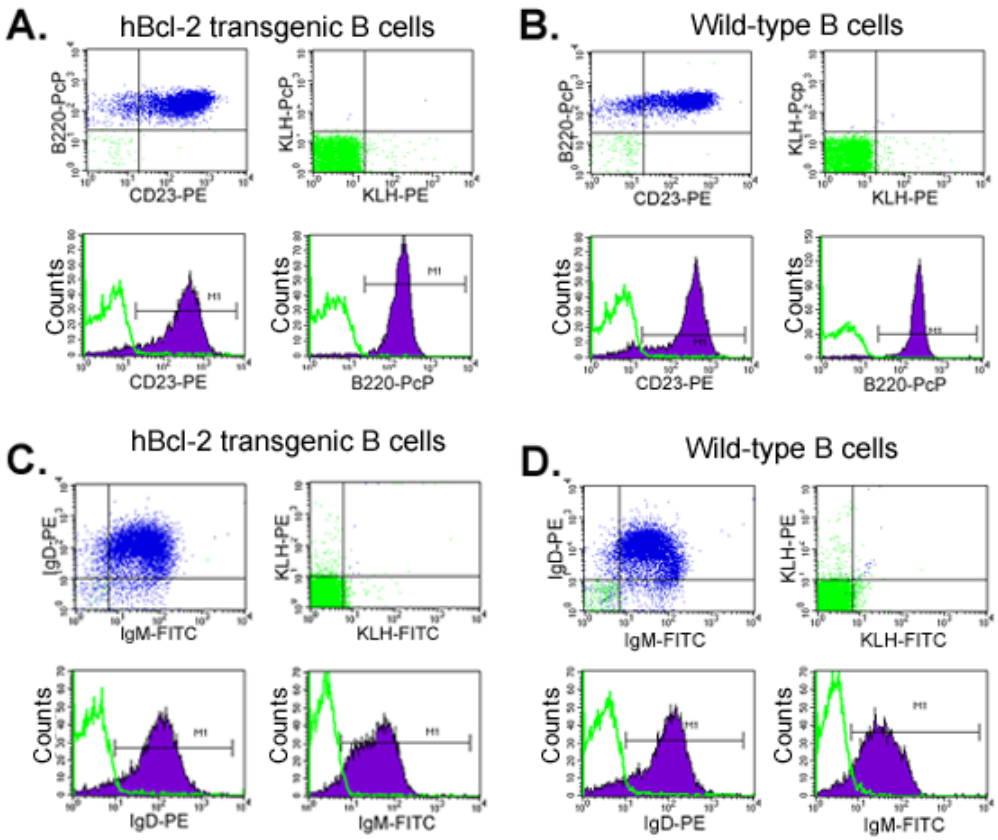

Fig. 3. **FACS cytometry analysis of hbcl-2 transgenic versus wild-type splenic B cell subsets.** Purified splenic B cells were stained with anti-IgM-FITC, anti-CD23-PE, anti-B220-PcP, anti-CD11b-APC, and anti-CD3ε-APC fluorophore-coupled antibodies. Samples were then analyzed by FACS cytometry, using Cell Quest software. The top left histogram of each panel shows dot plots of live cells stained with anti-CD23-PE versus anti-B220-PcP (A and B) and anti-IgD-PE versus anti-IgM-FITC (C and D) for purified splenic B cell populations from both hbcl-2 transgenic and wild-type mice. Isotype control dot plots, stained with anti-keyhole limpet hemocyanin (KLH) antibodies coupled to PE and/or FITC fluorophores are shown in the top right histogram of each panel. The cell classification criteria, according to expression of surface receptors, were taken from Hardy and Hayakawa (16). The expression or lack of expression of a specific protein is indicated by + or –, respectively; cell subsets, which either can or cannot express a specific protein, are designated +/- [noted in Fig. 4]. Positive expression boundaries are marked by either horizontal lines (M1) on the histogram plots, or right-hand, and upper quadrants for x and y axes respectively, marked on the dot plots. The relative strength of expression for a given protein is indicated by the number of + signs from one to four, ++++ being the highest level of expression observed in the expression range for the population. The majority of splenic B cells were mature B2 B cells (IgM^+, IgD^{+++}, $CD23^{++}$ and $B220^{+++}$ for both strains. The histogram plots show the intensity of staining with the specific antibodies designated on the x-axis (purple fills) with isotype control antibodies (green lines) on the same plots. Representative experiments are shown for staining on 17 B cell preparations from hbcl-2 transgenic and 7 wild-type litter-matched mice.

cells (CD23^{++}, CD21^{++}; region R2, Fig. 4) were 89% and 88%, respectively, in hbcl-2 transgenic and wild-type splenic B cell populations. Although CD23 expression levels appeared normal, CD21 levels on hbcl-2 transgenic B cells were reduced compared to wild-type B cells (median fluorescence intensity more than twofold less). The sum of the transitional (T2 and T1) and B1 B cell subpopulations was 7% for hbcl-2 transgenic and 5% for wild-type splenic B cell populations (Fig 4, top panels, regions R3 and R4; plots gated for live B220 positive cells).

In summary, hbcl-2 transgenic splenic B cells are comparable to wild-type splenic B cells in that the majority of cells express the surface receptor characteristics of mature B2 B cells. The main difference detected in the hbcl-2 B cell population compared with wild-type B cells was the absence/reduction of MZ B cells, as was also recently reported by Brunner and colleagues in a different strain of bcl-2 transgenic mouse (17). Importantly, the transgenic expression of bcl-2 does not lead to an accumulation of B cells that are IgM$^{-/-}$, consistent with evidence that bcl-2 expression alone is not sufficient for the persistence of mature B cells, which also

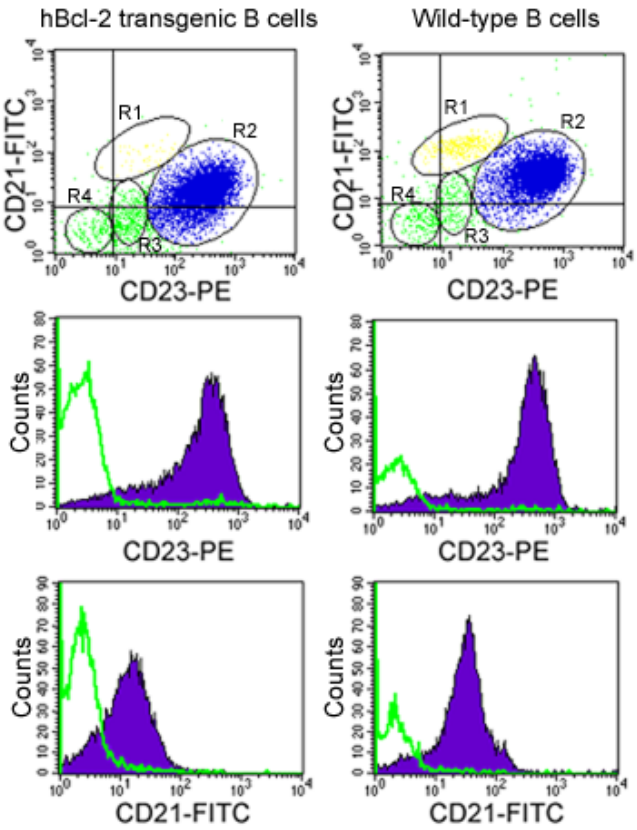

Fig. 4. **Marginal zone B cells were reduced in hbcl-2 transgenic splenic B cells.** Purified splenic B cells were stained with anti-CD21-FITC, anti-CD23-PE, anti-B220-PcP, anti-CD11b-APC, and anti-CD3ε-APC fluorophore-coupled antibodies. Samples were then acquired and analyzed for four-color staining using FACS cytometry, with Cell Quest software. The top two panels were gated on live B cells (using forward- and side-scatter profiles and positive B220 staining) and show dot plots of anti-CD21-FITC versus anti-CD23-PE staining for both hbcl-2 transgenic and wild-type B cells. The regions R1 to R4 designate recognized subsets of splenic B cells. R1 = Marginal zone (MZ) B cells (CD21^{+++}, CD23^{-}); R2 = Follicular B cells (CD23^{++}, CD21^{++}), R3 and R4 = Transitional, T2 or T1, and B1 subsets of B cells (CD21^{+}CD23^{+}, CD21$^{+/-}$CD23^{-}, CD21$^{+/-}$CD23$^{++/-}$, respectively). The lower four panels, also gated on live B cells, show the intensity of staining with specific antibodies (purple-fill histograms) and isotype control antibodies (green lines). Representative experiments are shown for staining on four separate spleen preparations.

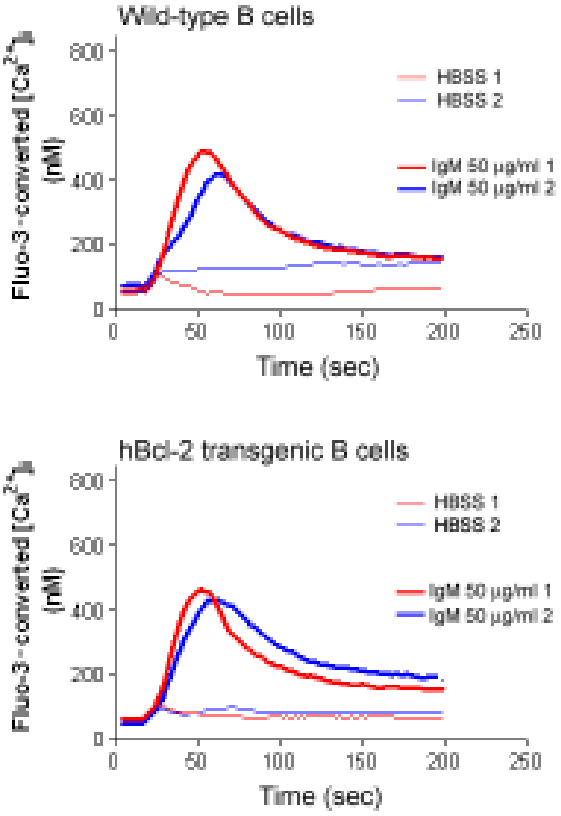

Fig. 5. Calcium flux responses in fresh hbcl-2 transgenic and wild-type B cells. Fluo-3–loaded B cells were plated in 96-well black-walled plates and allowed to settle on the bottom of the wells (10^6 cells/well). A FLEXstation fluorometer (Molecular Devices) was used to excite and take emission readings for individual wells from the bottom of black-walled clear-bottom 96-well assay plates. The numbers 1 and 2 denote replicate wells read in a single experiment. Baselines were recorded prior to the robotic addition of anti-IgM at 20 sec, followed by 5 µM ionomycin/10 mM EGTA/1 µM thapsigargin at 300 sec (to give a minimum fluorescence value for loaded cells), and 25 mM $CaCl_2$ at 600 sec (to give a maximum fluorescence value for loaded cells). Relative fluorescence unit traces (readings taken every 1.26 sec) were converted to calculated $[Ca^{2+}]_i$ values using the min and max values measured for each well with the formula given in *Methods and Protocols*. The converted $[Ca^{2+}]_i$ traces shown are for two wells each of wild-type versus hbcl-2 transgenic B cells. Negative control wells (HBSS buffer added at 20 sec) were run for both populations. Average peak height $[Ca^{2+}]_i$ values for wild-type B cells were HBSS controls 126 nM and anti-IgM 588 nM; for bcl-2 transgenic B cells, values were HBSS controls 106 nM and anti-IgM 446 nM. A representative experiment of three experiments performed on different B cell preparations is shown.

requires signaling through the pre-BCR or BCR during development (18).

Functional Responses in Freshly Isolated hBcl-2 Transgenic and Wild-Type B Cells

To determine if hbcl-2 transgenic B cells were functionally similar to wild-type B cells, we compared outputs following BCR ligation, including intracellular calcium ($[Ca^{2+}]_i$) flux, protein phosphorylation, upregulation of activation markers (CD86 and CD69), and gene expression changes (see below; Gene Expression Changes in Response to In Vitro Culture and Anti-IgM Stimulation). In the two populations, activation of the BCR with anti-IgM caused comparable $[Ca^{2+}]_i$ flux (Fig. 5), protein phosphorylation (Fig. 6), and upregulated expression of the activation markers CD69 and CD86 (Fig. 7). We also measured proliferation responses to LPS, anti-CD40, and IL-4, in addition to anti-IgM (Fig. 8) and examined migration responses to the chemokines SDF-1α and BLC, comparing hbcl-2 transgenic B cells to wild-type B cells (see below; Functional Responses in Cultured Human Bcl-2 Transgenic B Cells). Both ^{3}[H]-thymidine incorporation and cell count indicators of proliferative responses by hbcl-2 transgenic B cells in response to LPS, anti-IgM, anti-CD40, and IL-4 (singly and in pair combinations) were generally less than those in wild-type splenic B cells (Fig. 8). Importantly, LPS produced the greatest increase in ^{3}[H]-thymidine incorporation for both hbcl-2 transgenic and wild-type B cells over multiple experiments and was the only stimulus able to consistently increase hbcl-

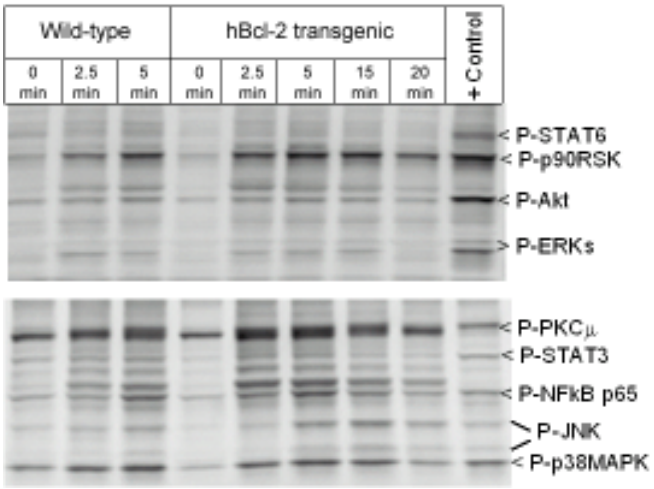

Fig 6. **Stimulation of protein phosphorylation following anti-IgM stimulation of fresh wild-type and hbcl-2 transgenic B cells.** B cells were plated in 12-well tissue-culture plates and stimulated according to AfCS protocol PP00000010. Briefly, 1.8 X 10^7 fresh wild-type or hbcl-2 transgenic B cells were stimulated with anti-IgM at 50 µg/ml for 0, 2.5, and 5 min or 0, 2.5, 5, 15, and 30 min, respectively, then lysed in SDS-PAGE sample buffer containing phosphatase inhibitors and processed for SDS-PAGE and Western blotting. hBcl-2 transgenic splenic B cells are slightly smaller than wild-type B cells (detected by FACS analysis of cell forward scatter), and protein yields from these cells averaged 6.9 µg and 9.9 µg per million cells, respectively. Equivalent amounts of protein, 20 µg per lane, were run on SDS-PAGE gels. Antibody mixes for probing Western blots were developed by the AfCS. The top blot was probed with phosphospecific antibody mix 1, which contained phosphospecific antibodies for stat-6, p90RSK, Akt, and ERK-1 and -2 (PS00000312; positive (+) control A, PS00000083). The lower blot was probed with phosphospecific antibody mix 2, which contained phosphospecific antibodies for PKCμ, Stat-3, NFκB, JNK, and p38, listed in order of decreasing protein size as observed on blots (PS00000334; positive (+) control B, PS00000085).

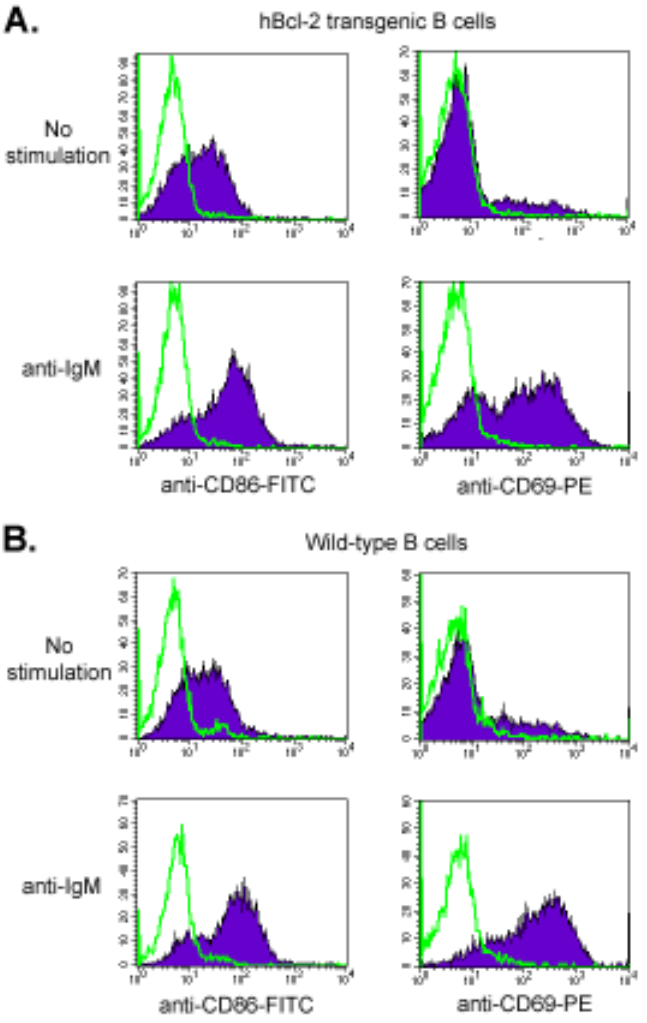

Fig. 7. **Upregulation of activation markers CD86 and CD69 in hbcl-2 transgenic versus wild-type B cells**. B cells were isolated and plated in 12-well culture dishes for 1 hr (PP00000010). B cells were then either nonstimulated or stimulated with anti-IgM (50 µg/ml). At 24 hr poststimulation, hbcl-2 transgenic (panel A) and wild-type (panel B) B cells were harvested and processed for antibody staining to detect expression of the activation markers CD69 and CD86. B cells were stained with anti-CD86-FITC and anti-CD69-PE antibodies and analyzed using FACS cytometry. Histogram plot analyses were gated for live cells and show specific fluorescent signals observed for either anti-CD69 or anti-CD86 staining, as listed on the x-axes (shown with purple-fill traces), and isotype controls (shown with green-line traces). Representative data from three separate experiments is shown.

2 B cell numbers within two days of stimulation. In contrast, for wild-type B cells, anti-CD40 alone or in combination with anti-IgM or IL-4 produced an increase in cell numbers above those obtained for unstimulated cells, which died within two days. The reduced proliferation response observed here with hbcl-2 transgenic B cells is consistent with previously described effects of overexpression of bcl-2, resulting in delayed transition of B cells from the quiescent state into the cell cycle (19).

Effects of Culture on B Cell Surface Marker Phenotype

To determine if the cultured hbcl-2 transgenic B cells maintained their surface phenotype during culture, we measured the expression of several surface markers on these cells. Human bcl-2 transgenic B cells cultured for up to six days essentially retained their surface phenotype, as assessed by expression of IgM, B220, and CD23 (Fig. 9). The percentage of cells determined positive for these markers averaged 85%, 99%, and 92% for fresh cells and 80%, 100%, and 76% for day 6 cultured cells for IgM, B220, and CD23, respectively, suggesting an apparent decline in CD23 expression in culture. Absolute fluorescence intensity values for cells stained on different days cannot be directly compared; however, the ratios of median fluorescence intensities for stains and isotype-matched controls gave an indication of relative levels of specific marker expression. The histograms shown (Fig. 9), and data from other experiments, suggested an overall reduction in CD23 expression after six days in culture compared with fresh cells (Fig. 4). The day 6 cultured cells showed a broader range of CD23 expression, with

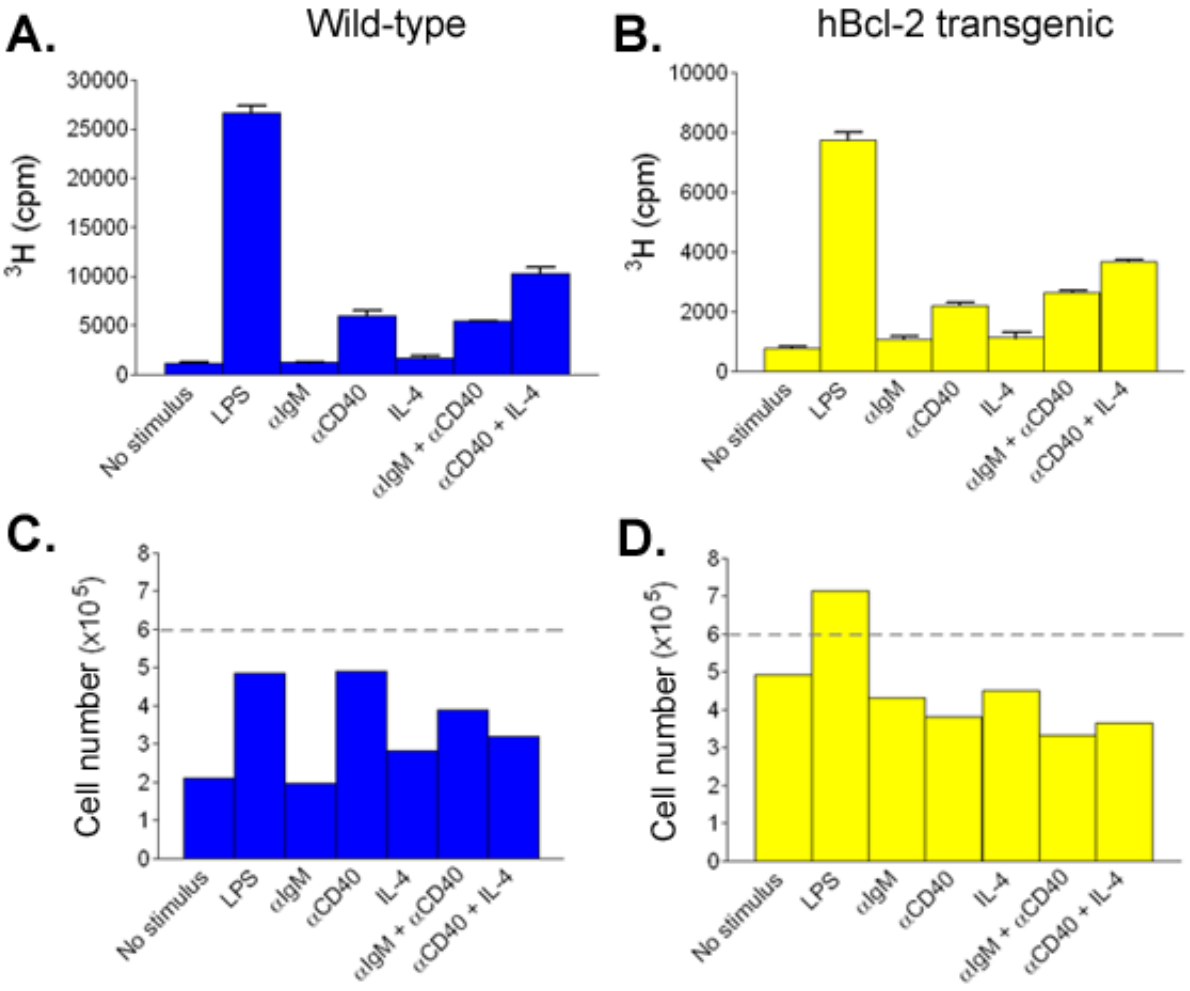

Fig. 8. **Proliferation responses of fresh wild-type and hbcl-2 transgenic B cells.** Freshly isolated wild-type (blue) or hbcl-2 transgenic (yellow) B cells were plated at 6 x 10^6/ml, 100 µl/well, in 96-well plates. Duplicate plates for ^{3}[H]-thymidine incorporation assays and hand counts were harvested separately on day 1 (graphs A and B) and day 2 poststimulation (graphs C and D), respectively. Stimuli used were LPS (20 µg/ml), goat polyclonal anti-mouse IgM (50 µg/ml), hamster IgM anti-CD40 (at 20 µg/ml), IL-4 (50 U/ml), and hamster IgM isotype control antibody, which had no significant effect (data not shown). For combinations of anti-IgM and anti-CD40 or anti-CD40 and IL-4, the same doses that were used for single ligand stimulation were used in the combination. A single experiment is shown, including average values for quadruplicate wells for ^{3}[H]-thymidine incorporation (±SD error bars) and average count values from duplicate wells (2 counts each) for hand counts in each experiment. Similar results were obtained in three separate experiments.

increased numbers of lower expressing cells; however, we did not investigate this observation further. Day 6 cultured hbcl-2 transgenic B cells had similar levels of chemokine receptor expression compared to freshly isolated wild-type B lymphocytes (data not shown), and hbcl-2 transgenic B cells sustained CXCR4 and CXCR5 expression for up to six days in culture (Fig.10). In accordance with this, migration responses to the chemokines BLC and SDF-1α were also sustained in cultured hbcl-2 transgenic B cells (day 1, 3, and 6) and were similar to those seen with fresh B cells (see below; Functional Responses in Cultured Human Bcl-2 Transgenic B Cells). CXCR5 was normally expressed at a higher level than CXCR4 (median fluorescence intensities usually 3- to 6-fold and 1.5- to 2-fold isotype control intensities, respectively). The majority of freshly isolated wild-type and hbcl-2 transgenic splenic B cells lacked expression of the activation markers CD69 and CD86 (Fig. 7). Culture of hbcl-2 transgenic B cells for up to six days in the absence of stimulation did not cause any increase in expression of these activation markers (see below; Functional Responses in Cultured Human Bcl-2 Transgenic B Cells).

Human bcl-2 was readily detected by fluorescence cytometry analysis of the hbcl-2 transgenic B cells, as expected, and its expression was maintained in culture, measured at up to six days (data not shown). Mature resting B cells also expressed endogenous mouse bcl-2 protein (8), and we observed that mouse bcl-2 was expressed at similar levels in hbcl-2 transgenic and wild-type B cells (data not shown). Only B cells from hbcl-2 transgenic mice expressed human bcl-2. As a consequence, other contaminating cell types (<5%) failed to survive in culture. Hence, the purity of hbcl-2 transgenic B cells increased from greater than 96% (Table 1) at isolation to 100% after one to six days in culture (data not shown). In conclusion, hbcl-2 transgenic expression is maintained in the hbcl-2 B cells in culture, imparting a selective survival advantage on these cells versus any contaminating immune cells. This allows the establishment of pure cultures of resting mature splenic B cells.

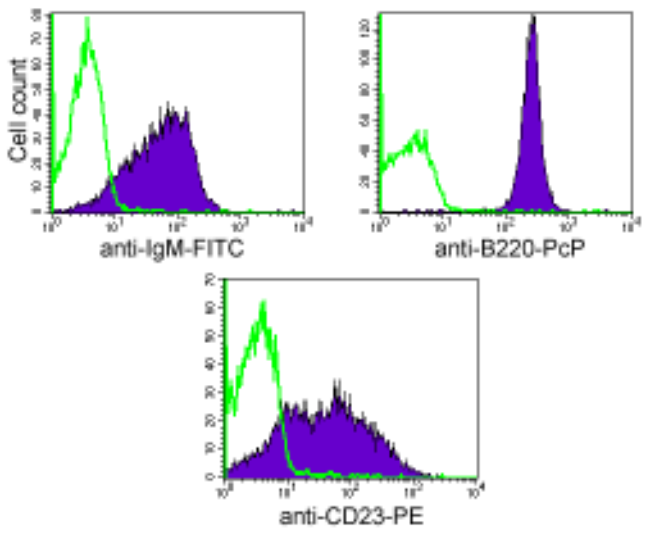

Fig. 9. **Surface phenotype of cultured hbcl-2 transgenic B cells.** Purified splenic hbcl-2 transgenic B cells were cultured for 6 days then harvested and stained with anti-IgM-FITC, anti-CD23-PE, anti-B220-PcP, anti-CD11b-APC, and anti-CD3ε-APC fluorophore-coupled antibodies. Samples were acquired and analyzed for four-color staining by FACS cytometry, using Cell Quest software. The histograms shown were gated on live cells (using forward and side scatter profiles), and plots of fluorescence intensities for specific stains (listed on x-axes, in purple fill) and isotype control stains (green lines) are shown.

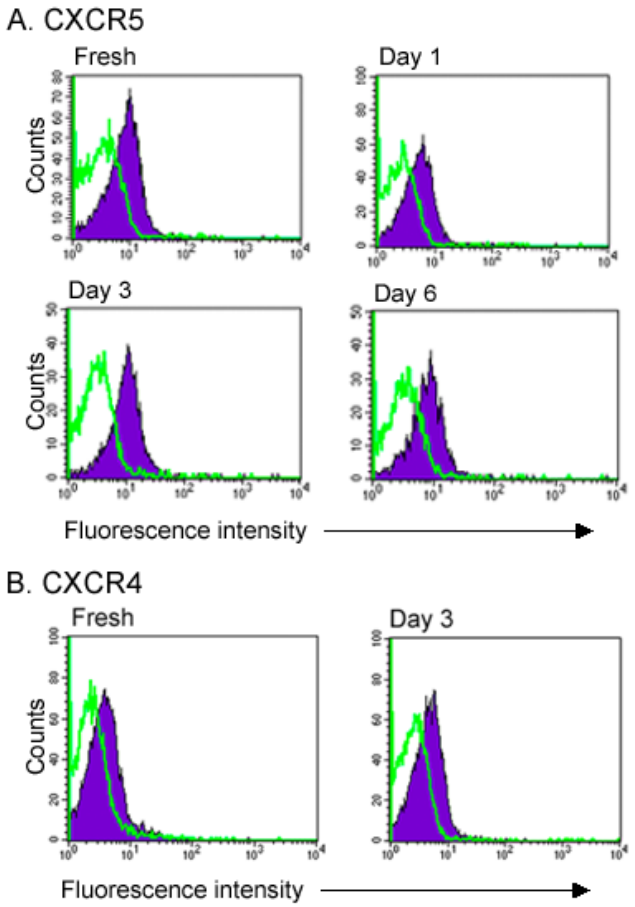

Fig.10. **Chemokine receptor expression of cultured hbcl-2 transgenic B cells.** Expression of the chemokine receptors CXCR5 and CXCR4 on fresh versus cultured hbcl-2 transgenic B cells was examined using FACS cytometry analysis. (A) Anti-CXCR5-PE fluorescence intensity, shown on the x-axis, showed the expression of the chemokine receptor CXCR5 (purple-filled histograms) was stable in hbcl-2 transgenic B cells, cultured for up to six days in 10% FBS. The green-line histograms show fluorescence intensity using isotype-matched fluorophore-coupled negative control antibodies. Antibodies were used at 1 μg/10^6 cells, and 0.25 x 10^6 cells were stained in 100 μl. (B) CXCR4 staining on fresh and day 3 cultured hbcl-2 transgenic B cells was also stable. Anti-CXCR4-FITC fluorescence intensity is shown in purple fills, with isotype-matched controls shown as green lines. Antibodies were used at 1 μg/10^6 cells, and 0.25 x 10^6 cells were stained in 50 μl of buffer (using the buffers described in AfCS protocol PP00000018). Representative staining for three hbcl-2 splenic B cell preparations (A) and (B) is shown.

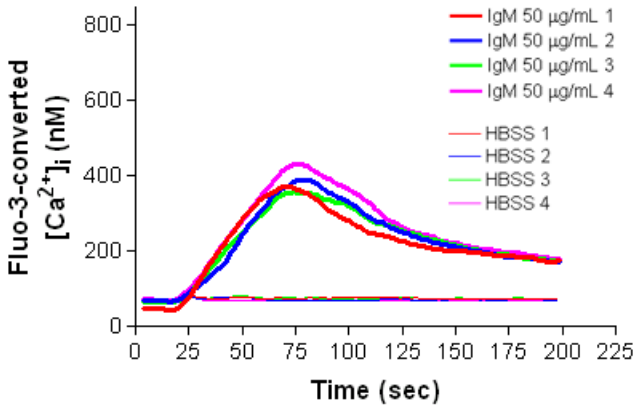

Fig. 11. **Calcium flux in response to anti-IgM stimulation in cultured hbcl-2 transgenic B cells.** Cultured day 6 hbcl-2 transgenic B cells were harvested and counted. Viable cell number was assessed by trypan blue exclusion. Cells were loaded with fluo-3 indicator dye for 30 min in tubes then plated into 96-well plates and allowed to equilibrate for 30-min at 37°C prior to assay. Anti-IgM or vehicle control (HBSS buffer) was robotically added to quadruplicate wells (individual wells denoted by numbers 1 to 4) in the assay plate simultaneously. Quadruplicate samples for a single experiment are shown; similar $[Ca^{2+}]_i$ flux responses were observed in three separate experiments.

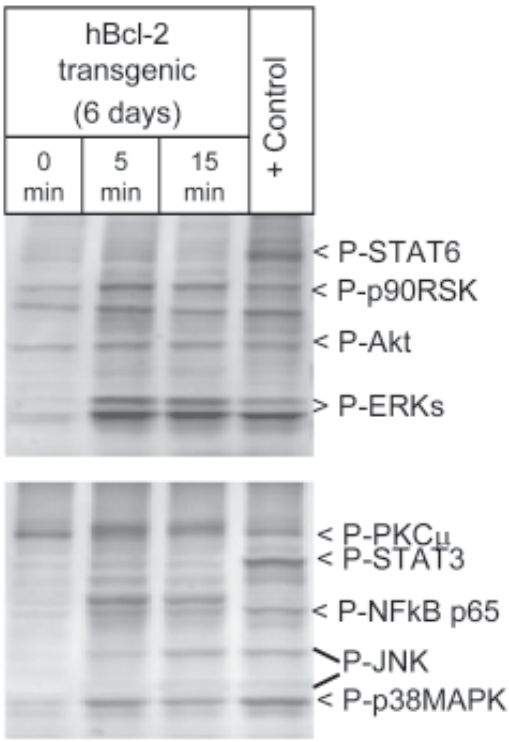

Fig. 12. **Protein phosphorylation responses to anti-IgM stimulation in cultured hbcl-2 transgenic B cells.** Cultured day 6 hbcl-2 transgenic B cells were harvested and counted. Viable cell number was assessed by trypan blue exclusion. Viable cells were replated in 12-well tissue-culture plates and rested for 1 hr prior to stimulation with anti-IgM at 50 µg/ml. Cells were stimulated according to AfCS protocol PP00000010. Briefly, 18 x 10^6 viable cultured hbcl-2 transgenic B cells were stimulated for 0, 5, or 15 min and then lysed in SDS-PAGE sample buffer, containing protease and phosphatase inhibitors, and processed for SDS-PAGE and Western blotting. Equivalent amounts of protein, 20 µg per lane, were run on SDS-PAGE gels. Antibody mixes developed by the AfCS (see Fig. 5 legend) were used to probe blots. Mix 1 contained phosphospecific antibodies for stat-6, p90RSK, Akt, and ERK-1 and -2. Mix 2 contained phosphospecific antibodies for PKCµ, Stat-3, NFκB, JNK, and p38 (listed in order of decreasing protein size as observed on blots).

Functional Responses in Cultured hBcl-2 Transgenic B Cells

To determine if hbcl-2 transgenic B cells retained their functional responses in culture, we first measured signaling responses to BCR stimulation in cells cultured for up to six days. These included intracellular calcium flux and the phosphorylation of signaling proteins, including PKCµ, Akt, ERK1/2, p38MAPK, JNK, p90RSK, NFκB p65, STAT3, and STAT6. Functional responses by hbcl-2 transgenic B cells were sustained for up to six days in culture (Fig. 11 and 12). The activation markers CD69 and CD86 were also upregulated in response to anti-IgM in cultured hbcl-2 transgenic B cells (Fig. 13). B cell proliferative responses to anti-IgM, IL-4, and/or anti-CD40, or LPS were determined by measurement of ^{3}[H]-thymidine incorporation and by viable cell counts assessed by trypan blue exclusion (Fig. 14). Day 6 cultured hbcl-2 transgenic B cells showed the same

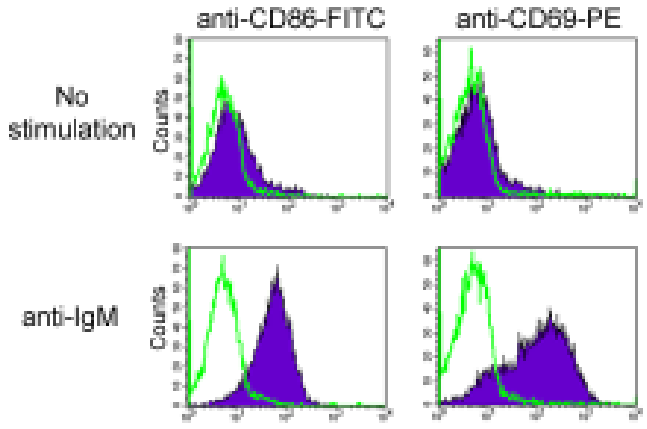

Fig. 13. **Upregulation of activation markers CD86 and CD69 in response to anti-IgM stimulation in cultured hbcl-2 transgenic B cells.** hBcl-2 transgenic B cells were cultured for six days in medium containing 10% FBS then harvested and counted to determine viability (using trypan blue exclusion). Cells were replated in 24-well plates (1 x 10^6/well in 1 ml) and stimulated with anti-IgM (50 µg/ml) overnight. The next day, cells were harvested and stained with anti-CD86-FITC and anti-CD69-PE (shown in purple-filled histograms) or appropriate fluorophore-coupled isotype negative control antibodies (shown with green-line histograms). Stained cells were analyzed by FACS cytometry; the histograms shown were gated for all live cells. The results shown are from a single experiment, replicate experiments (2) produced similar results.

relative ^{3}[H]-thymidine incorporation responses to these specific stimuli that were observed with fresh hbcl-2 transgenic or wild-type B cells. As with fresh hbcl-2 transgenic B cells, day 6 cultured cells showed no increase in cell numbers at two days poststimulation for any stimulus (anti-IgM, anti-CD40, and IL-4) other than LPS (Fig. 8 and 14). Finally, migration was assessed in response to the chemokines SDF-1α and BLC (the ligands for CXCR4 and CXCR5, respectively) and was also not diminished by culture of these cells (Fig. 15).

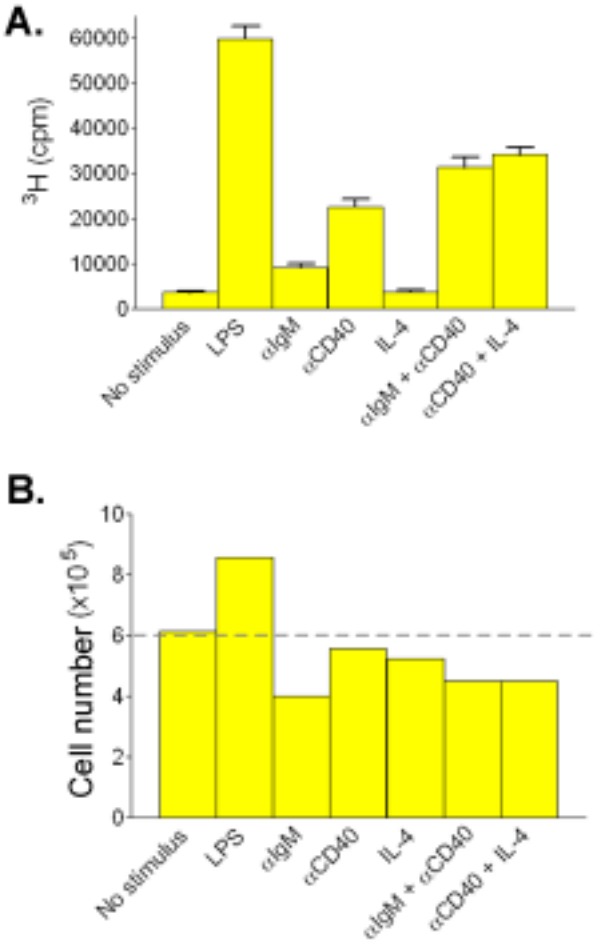

Fig. 14. **Proliferation responses in cultured hbcl-2 transgenic B cells.** Cultured day 6 hbcl-2 transgenic B cells were harvested, viability counts were taken, and cells were replated at 6 x 10^{6} viable cells/ml, 100 µl/well, in 96-well plates. Duplicate plates for ^{3}[H]-thymidine incorporation assays and hand counts were harvested separately on day 1 (A) and day 2 poststimulation (B), respectively. Stimuli used were LPS (20 µg/ml), goat polyclonal anti-mouse IgM (50 µg/ml), hamster IgM anti-CD40 (at 20 µg/ml), IL-4 (50 U/ml), and hamster IgM isotype control antibody, which had no significant effect (data not shown). For combinations of anti-IgM and anti-CD40 or anti-CD40 and IL-4, the same doses that were used for single ligand stimulation were used in the combination. A single experiment is shown; average values for quadruplicate wells for ^{3}[H]-thymidine incorporation (±SD error bars) and average count values from duplicate wells for hand counts in each experiment are given. Similar results were obtained in three separate experiments.

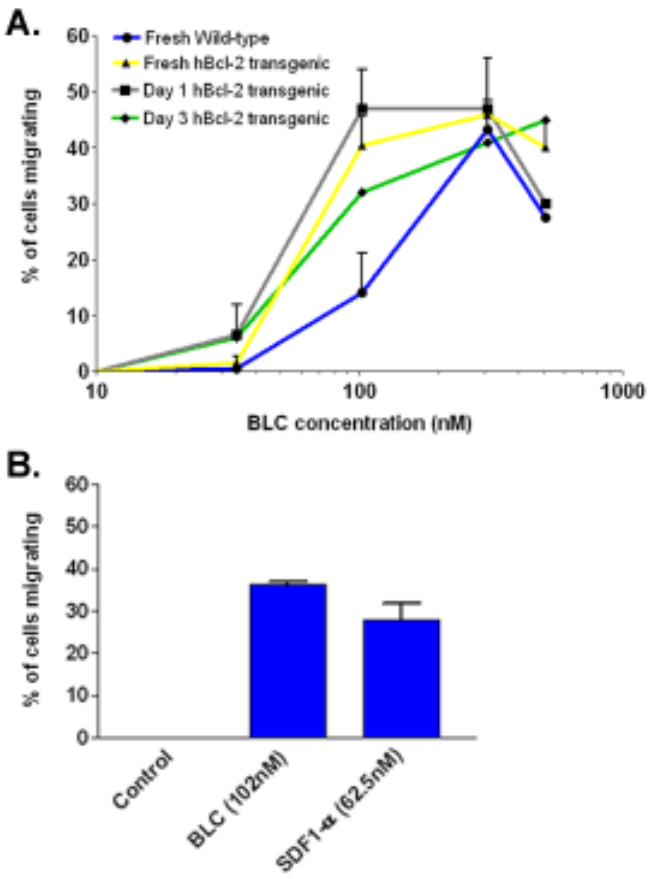

Fig. 15. **Cultured hbcl-2 transgenic B cells migrated in response to BLC (CXCL13), the ligand for CXCR5, and SDF-1a (CXCL12), the ligand for CXCR4**. (A and B) Transwell migration experiments were performed over 4-hr periods with 1 x 10^{6} cells per sample. Average migrations from multiple experiments are shown. SEM error bars are for three or more repeat experiments; within each experiment, transwell samples were run in duplicate. The percentage of cells migrating was calculated using the positive control maximum (i.e., cells pipetted directly into the lower chamber of the transwell and harvested alongside test samples). (A) Graphs show dose response data for fresh (wild-type or hbcl-2 transgenic) and cultured (hbcl-2 transgenic) B cells to the chemokine BLC. (B) The histogram shows the responses of day 6 cultured bcl-2 transgenic B cells migrating to near optimal doses of BLC (102 nM = 1 µg/ml) or SDF (62.5 nM = 500 ng/ml). Graphs show the averages of three experiments (±SEM) on different B cell preparations (duplicate transwells per experiment), except (A) hbcl-2 day 3, which shows the average of two experiments (hence, no error bars given).

Gene Expression Changes in Response to In Vitro Culture and Anti-IgM Stimulation

The observation that cultured hbcl-2 transgenic B cells essentially maintained mature B cell surface marker phenotypes and showed comparable functional responses to freshly isolated transgenic and wild-type B cells indicated that the cultured cells were a stable population. To further examine the suitability of cultured hbcl-2 transgenic B cells as an alternative signaling model for primary B cells, we employed microarray analyses of gene expression profiles in these cells. Gene expression changes induced by culture and

the effect of prolonged culture on transcriptional responses to anti-IgM treatment were assessed. A few probes showed reproducible changes in expression between day 0 and day 1 or day 0 and day 3 of culture. Of these, we identified two clusters of differentially expressed probes with statistically significant associations with biological functions or processes, using CLASSIFI analysis. One of the clusters was enriched with probes from hemoglobin genes, which were downregulated following one and three days of culture (Fig. 16A and C). The downregulation of hemoglobin genes might be due to the gradual disappearance of contaminating reticulocytes from B cell cultures with time. The second cluster of probes, mostly from genes encoding enzymes in the glycolysis pathway, showed consistent upregulation only in hbcl-2 transgenic B cells cultured for three days (Fig. 16A and B). Glycolysis regulation is required for cell survival (20-22). Indeed, the proapoptotic BCL-2 family member, BAD (which can be sequestered by antiapoptotic BCL-2 or BCL-X_L), has recently been shown to occupy an unanticipated role in integrating signaling pathways for glucose metabolism and apoptosis (23). In conclusion, the upregulation of genes encoding glycolysis pathway enzymes in hbcl-2 transgenic B cells after three days could be contributing to the survival of these cells during prolonged culture.

hBcl-2 transgenic B cells had robust, and generally consistent, transcriptional responses to anti-IgM stimulation following prolonged culture. A common set of 228 array probes showed at least an average of twofold expression changes in response to 2 hours of anti-IgM treatment in B cells that had been cultured for 0 days, 1 day, 3 days, or 6 days. Of the 121 probes from known genes, 80 array probes representing 65 genes were upregulated, while 41 array probes representing 38 genes were downregulated (see Table 2, supplemental Excel file, for complete gene list). To evaluate the effect of prolonged culture on the transcriptional response to anti-IgM, we compared anti-IgM–induced expression changes of 1007 array probes in B cells cultured for different numbers of days. These probes exhibited at least twofold expression changes when B cells were stimulated with anti-IgM for 2 hours at one or more time points during culture. The majority of the probes showed similar expression changes whether the cells were stimulated on day 0, 1, 3, or 6, as visualized by hierarchical clustering (data not shown). Only 10 out of the 1007 probes were differentially expressed at a false discovery rate of 8% between different time points during culture, as determined by Multiclass SAM analysis (Fig. 17). Many of these 10 probes, such as those representing cAMP responsive element modulator, calmodulin 1, golgi phosphoprotein 3, and fatty acid binding protein 5, showed different expression responses to anti-IgM stimulation in B cells cultured for 0 days versus in B cells cultured for 6 days. Whether these differences are the results of prolonged culture is unknown, and their relevance to cellular response to anti-IgM is undetermined.

To determine whether anti-IgM–induced gene expression changes in hbcl-2 transgenic B cells were similar to those induced in freshly isolated wild-type B cells, we

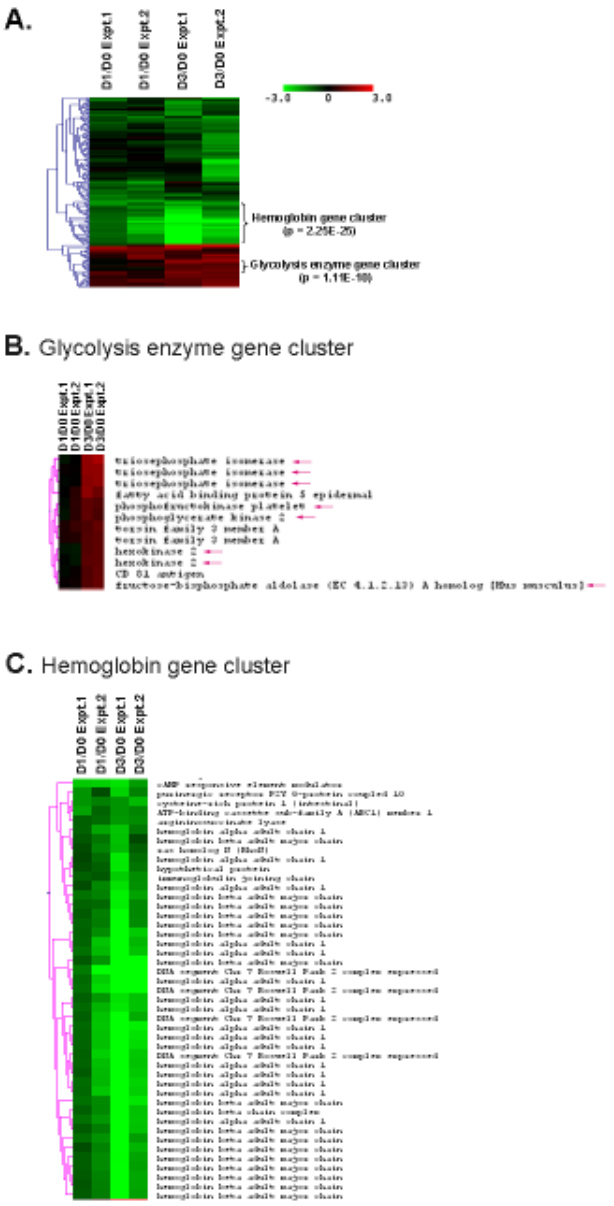

Fig.16. **Gene expression changes in hbcl-2 transgenic B cells during in vitro culture.** (A) A dendrogram of hierarchically clustered gene expression changes of hbcl-2 transgenic B cells after one and three days of in vitro culture. The gene expression changes were calculated as the ratio of expression level at day 1 or day 3 (normalized and background subtracted cy5 intensity) versus that at day 0 (normalized and background subtracted cy3 intensity) in $\log_2$. Only probes with duplicate measurements and an average of twofold or more changes in response to one or three days of culture were included. Two or three replicate data sets were presented for each time point in culture. Each replicate data set is labeled as Expt. Data sets for B cells cultured for one and three days are labeled as D1/D0 and D3/D0, respectively. CLASSIFI analysis identified two clusters of probes that showed significant association with glycolysis and hemoglobin. (B) The cluster enriched with probes from genes encoding glycolysis enzymes was upregulated only in hbcl-2 transgenic B cells cultured for three days. Glycolysis enzyme genes are indicated by red arrows. (C) The cluster enriched with probes from hemoglobin genes was downregulated in both hbcl-2 B cells cultured for one day and those cultured for three days.

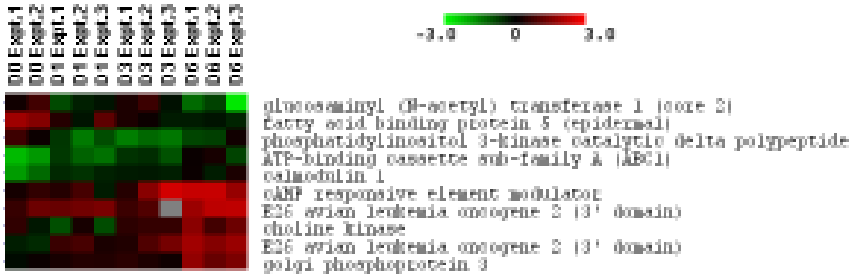

Fig.17. **Only a few probes showed different expression responses to anti-IgM stimulation in hbcl-2 transgenic B cells cultured for different numbers of days.** The 10 probes were identified by SAM analysis as differentially responded to anti-IgM (AIG) treatment among hbcl-2 transgenic B cells that had been cultured for 0 days (D0), 1 day (D1), 3 days (D3), and 6 days (D6) at a false discovery rate of 8%. A dendrogram of hierarchically clustered expression changes of the probes in hbcl-2 transgenic B cells in response to AIG stimulation is shown. The gene expression change was calculated as the ratio of the expression level in stimulated cells (normalized and background subtracted cy5 intensity) versus that in the time-matched control cells (normalized and background subtracted cy3 intensity) in $\log_2$. There are two or three replicate data sets for each time point, where each replicate data set is labeled as Expt. The color scale of expression changes is the same as that in Fig. 16. The gray spot represents blank measurements.

compared the data with those in wild type B cells stimulated for 1 hour, 3 hours, and 6 hours with anti-IgM measured in another experiment. Gene expression changes relative to time-matched control cells were determined in a similar manner as for the hbcl-2 transgenic B cells. A total of 1416 probes showed twofold or more anti-IgM–induced expression changes in either hbcl-2 transgenic or wild-type B cells, and their expression changes were generally similar in direction (up- or downregulation) in both types of B cells. By examining the dendrogram of hierarchically clustered expression changes of the 1416 probes (data not shown), we found that the magnitude of expression change in hbcl-2 transgenic B cells (2 hour–stimulated with anti-IgM) was generally greater than that in 1 hour–stimulated wild-type B cells and comparable to that observed in 3 or 6 hour–stimulated wild type B cells, as would be expected if hbcl-2 transgenic and wild-type B cells have similar transcriptional responses to anti-IgM. Finally, the results regarding anti-IgM–stimulated gene expression changes for both cell types were generally consistent with historical studies of wild-type B cells (24-26).

Targeted Manipulation of Signaling Protein Expression

To manipulate signaling protein expression, the AfCS plans to utilize plasmid-based shRNA- and/or siRNA-mediated RNAi or RNA antisense. These methods will require transfection or viral transduction to introduce exogenous DNA or RNA into the model cell system. Unfortunately, primary B cells are resistant to transfection by electroporation or by chemical techniques. Furthermore, transduction with Moloney-based retroviral systems has been achieved only in B cells that have been prestimulated and that are actively cycling (27, 28). However, a subset of retroviruses, the lentiviruses, can infect nondividing cells. HIV is one type of lentivirus, and recently, HIV-based lentiviral systems have been developed to transduce noncycling cells (29, 30).

To examine transduction of B cells by lentivirus, we tested two related and overlapping systems (see *Methods and Protocols*) for the production of nonreplicating lentivirus vectors. The data shown are for cells infected with lentivirus carrying green fluorescence protein (GFP) as a marker for successful transduction. Infection of 293T cells with unconcentrated lentivirus at a density of approximately 1 x 10^6 virus particles/ml, using a multiplicity of infection (MOI) of 5, caused infection of >95% of cells. By concentration of the lentivirus produced using Centricon filtration units, we obtained viral titers of 10^8 to 10^9 infectious units/ml, as assessed by transduction of fresh 293T cells, which allowed infection of target cells at higher virus densities and MOIs. However, in multiple experiments, we detected minimal transduction (~3%) of unstimulated fresh B cells from either hbcl-2 transgenic (Fig. 18A) or wild-type mice (data not shown), even with MOIs up to 50, at densities of >3 x 10^7/ml. Stimulation of B cells with an activation/proliferation promoting ligand, such as LPS or anti-CD40, for one day prior to infection produced a modest increase in expression of GFP relative to nonstimulated cells. In the experiment shown, approximately 6% of cells were transduced, and median fluorescence intensity of GFP in the transfected cell population increased by approximately 1.5X compared with noninfected cells (Fig. 18B). Of note, the same virus stock was successfully used to efficiently transduce the pro-B cell line Ba/F3 at >90% (Fig. 18C).

In other studies, success with primary T and B lymphocytes (human or mouse) has mainly been limited to infection of cells activated by cytokines and/or growth factors, suggesting that transduction of noncycling lymphocytes is inefficient (14, 15, 31-34). Lentivirus systems have been used successfully to transduce siRNA into T lymphocytes, but all published reports used T cells that were activated in vitro by mitogen or antigen and were subsequently stimulated with IL-2 (14, 15). Lentivirus vectors carrying modified promoters (human EF-1α, and improved biosafety designs) increased transduction efficiencies for some human hematopoietic cells (31), and relatively efficient transduction (>20%) of activated primary human B cells was reported with other lentivirus vector systems (32, 33). Rossi (34) recently reported higher levels of lentiviral-mediated transduction of mouse B cells stimulated with either LPS or anti-CD40, using pHR'CMV-eGFP-based lentivirus vectors. Early in our studies, on a

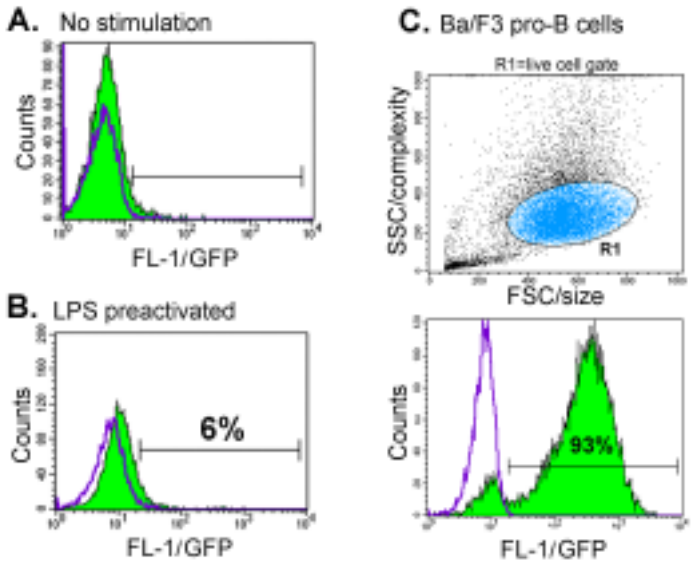

Fig.18. Lentivirus infection of hbcl-2 transgenic B cells compared with the pro-B cell line Ba/F3. Nonstimulated and one day LPS (10 μg/ml) preactivated hbcl-2 transgenic B cells (A and B) and log phase Ba/F3 pro-B cells (C) were infected with the same batch of concentrated eGFP carrying lentivirus (made using pFUGW vector plasmid) at an MOI of 50, in the presence of 4 μg/ml polybrene. Cells were returned to culture and harvested for FACS cytometry analysis at three days postinfection. Analysis of the proportion of cells transduced and expressing GFP was performed on live cells (R1 = region 1, determined by forward and side scatter properties) for each population; R1 dot plot data is not shown for B cells (A and B) but is shown for Ba/F3 cells (C). Successful transduction was measured in terms of relative eGFP expression, compared with autofluorescence for noninfected control cell populations. Cells exposed to virus are shown in green-fill histograms, negative/mock controls shown with purple line histograms (underwent identical experimental treatments with the exception of virus addition). Representative results from multiple experiments are shown.

number of occasions, we achieved levels of GFP expression comparable to those reported by Rossi, but our results were inconsistent, and this GFP expression was lost even though our virus preparations sustained transduction of control cells. When expression of GFP by B cells was observed in these experiments, it was a uniform increase in expression by all B cells, rather than by a subset, suggesting that this might represent an artifact of adsorption of GFP debris carryover from the 293T packaging cells (even though virus preparations were centrifuged and filtered prior to use; see *Methods and Protocols*). However, because we stopped seeing this phenomenon, and changed methods of virus production, we could not further test its origin. We conclude that B cells are, at the least, much more resistant to transduction using the current lentivirus vectors and methods than are other primary leukocytes (for which we have successfully obtained high efficiency transduction, i.e., activated primary T cells and bone marrow–derived macrophages).

In summary, we found that cultured splenic hbcl-2 transgenic B cells provided an excellent model for signaling studies in a primary mature B cell, despite being relatively refractory to gene transfection or transduction. Thus, hbcl-2 transgenic splenic B cells meet the following criteria as a useful model for the study of signaling:

i) Large numbers of B cells can be obtained from a single hemizygous spleen.
ii) B cells have extended survival in culture, with high viability in the absence of stimulation/activation.
iii) B cell purity improved with culture (through loss of the ~5% of contaminating cell types).
iv) The cultured B cells provided a stable platform for experiments examining the regulation of gene expression using RNA arrays.
v) All surface phenotypes and functional responses tested were essentially maintained in cultured hbcl-2 transgenic cells for up to six days.

Despite the inability to manipulate hbcl-2 transgenic B cells for signaling studies using the current methods for RNAi, this model system does open up many exciting alternative avenues for investigation. In addition to increasing the possibilities for microarray studies of long-term gene expression changes following single or dual ligand stimulations, the possibilities with other tools of signal transduction exploration, including the use of chemical inhibitors, knock-out mice, and the measurement of functional responses following ligand stimulations, can also be greatly expanded by using the hbcl-2 transgenic background in the B cells.

Methods and Protocols

Mice

Hemizygous bcl-$2^{+/TGN}$ (Eμ-bcl-2-22) transgenic mice were originally obtained from JAX Mice (http:// jaxmice.jax.org, Jackson Laboratory, stock no. 002319) and were maintained by breeding with C57BL/6 (B6) mice at the vivarium at the San Francisco VA Medical Center. These mice are currently maintained by JAX Mice only as frozen embryos. Hemizygous offspring were identified by using a PCR specific for the C-terminus of the human bcl-2 gene.

Genotyping by PCR

Genomic DNA from mouse tail ends (~5 mm length) was prepared by using Qiagen DNA extraction kits (QIAamp DNA mini kit, cat. no. 51306). Extracted DNA was tested for the presence of the human bcl-2 transgene and as a positive control, mouse IL-2, by using the Jackson Laboratory protocol for TgN(BCL2)22WEHI (http:// jaxmice.jax.org, genotyping protocol for stock no. 002319) using Amplitaq Gold (Roche, cat. no. 1699121) and a Gene Amp PCR System 9600 thermal cycler (Perkin-Elmer). This procedure identified both the PCR products for hbcl-2 (170 bp) and for mouse IL-2 (315 bp).

Hemizygotes made up 50% of litters, consistent with previous demonstration that the transgene is inserted at a single (unidentified) site in this line (5).

Isolation of B Cells

The AfCS protocol, *Isolation of Resting B Lymphocytes from One or More Groups of Four Mouse Spleens* (PP00000001), was used with the adjustment that one Bcl-$2^{+/TGN}$ spleen was processed as the equivalent of two B6 spleens. We avoided the use of Fc receptor (FcR) blocking antibodies during magnetic bead labeling, because we did not want the B cells exposed to these reagents.

Staining Cells for FACS Analysis

The AfCS protocol, *Characterization of Cells by Flow Cytometry* (PP00000018), was used. For staining of intracellular proteins such as bcl-2, we followed the manufacturer's instructions (BD kit, cat. no. 556357). Briefly, cells were manipulated at 4°C in PBS/2 mM EDTA/0.5% BSA. Fc receptor blocking and staining of cell surface receptors was done prior to fixing and permeabilization for staining of human or mouse bcl-2, and blocking agents were included during staining with fluorophore-coupled primary antibodies. The intensities of fluorescence staining signals obtained using antigen-specific antibodies directly coupled to fluorophores were always compared with signal intensities obtained using isotype-matched negative controls (e.g., antibodies raised against keyhole limpet hemocyanin [KLH]).

B Cell Culture

Isolated B cells were cultured in either Supplemented Iscove's Modified Dulbecco's Medium (SIMDM, AfCS solution protocol ID PS00000056) or SIMDM with 10% FBS (Hyclone), as specified. Cells were cultured at a density of 6 x 10^6/ml, plating 3 ml, 0.6 ml, or 0.1 ml in 6-well, 24-well, or 96-well Costar ultralow plates, respectively (Fisher Scientific cat. no. 07-200-601, 07-200-602, and 07-200-603). For the culture of cells for more than three days, half of the culture medium was replaced with fresh medium on days 3 and 6 of culture. Cells were not cultured for longer than nine days.

Ligands

AfCS ligands anti-IgM, SDF-1α, BLC, anti-CD40, and IL-4 were made up according to AfCS protocols (PL00000006, PL00000003, PL00000005, PL00000001, PL00000004). Lipopolysaccharide (LPS) from *Escherichia coli* 0111:B4 was purchased from Sigma-Aldrich (cat. no. L-4391). A stock solution of LPS was made by dissolving 1 mg (contents of 1 vial) in 1X PBS-0.1% BSA (PS00000082) to give a 1 mg/ml solution, which was aliquoted and stored frozen at –20°C.

Calcium Assay

Ninety-six–well plate calcium (Ca^{2+}) flux assays were performed using the FLEXstation (Molecular Devices) with modifications of two AfCS protocols PP00000011 (for suspended B cells) that is used with the Fluoroskan Ascent), and PP00000210 (for adherent RAW264.7, that is used with the FLEXstation). The FLEXstation, when operating on flex mode for robotic additions of ligands from a compound plate, excites and reads the assay plate from the bottom. Thus, to convert fluorescence units into calculated $[Ca^{2+}]_i$ values requires an equation that takes into account cell numbers being analyzed (given in PP00000210). This is in contrast to the measurements taken for suspended B cells using the Fluoroskan Ascent operated for a top read/total well assessment. Hence**,** modifications were made to load and plate nonadherent B cells and use minimum (extracellular calcium depleted, PS00000607) and maximum (excess extracellular calcium added, PS00000608) readings obtained using the procedure designed for assessments on adherent RAW 264.7 cells (PP0000210) to allow calculation of $[Ca^{2+}]_i$ values, assuming a K_d of 390 nM for fluo-3. Briefly, cells were loaded with the Ca^{2+} indicator dye, fluo-3 (3 μM, Molecular Probes), in the presence of pluronic F-127 in SIMDM medium. Cells were loaded for 30 minutes with fluo-3 in a polypropylene tube in the dark at room temperature and mixed during loading by inverting the tube every 10 minutes. After loading, cells were pelleted, resuspended in assay buffer (HBSS-BSA, PS00000032), plated into 96-well assay plates (1 x 10^6 cells/well, in 75 μl) for use in the FLEXstation, and then placed in a humidified incubator at 37 °C. Cells were left for 1 hour after plating to allow equilibration and settlement of cells to the bottom of the wells of the assay plate before Ca^{2+} flux assays were performed.

Phosphoproteins

Samples were prepared, run on SDS-PAGE gels, and Western blotted according to AfCS Protocol PP00000010. Western blots were probed with antibody mixes developed by the AfCS, PS00000312 and PS00000334, and with positive control samples, PS00000083 and PS00000085**,** respectively, for comparison.

Proliferation Assay

Freshly isolated splenic B cells (prepared using AfCS Protocol PP00000001) in MACS buffer (PBS/2 mM EDTA/0.5% BSA, PS00000001) or cultured B cells were harvested and resuspended in SIMDM (PS00000056) for assay at 6 x 10^5 cells/well. Ligands were added at the desired concentration to give a final volume of 200 μl/well, then 10 μl (1 μCi) of [^{3}H]-thymidine (Perkin-Elmer, cat. no.NET221X) was added per well, and assay plates were incubated overnight in a humidified atmosphere at 37 °C with 5% CO_2. The next day, plates were either harvested immediately and counted using a 96-well plate harvester/β-counter system (MicroBeta Tomtec, Perkin-Elmer) or were frozen for harvesting at a later time.

Migration Assay

Transwell migration assays were performed using 5 μm transwell inserts (Costar) suspended by the outer rim within individual wells of 24-well ultralow plates (Costar), so that B cells did not stick to the plates after migration through

the transwells into the lower well; this was a problem with some tissue culture–treated dishes. Use of the ultralow adhesion plates allowed all the migrating cells to be harvested for counting with minimal stress. The wells of the 24-well ultralow plates were filled with 0.6 ml of assay medium, SIMDM (PS00000056), with or without chemokine added. 1 x 10^6 B cells were added in 100 µl to the top transwell, which was placed in contact with the medium in the wells of the 24-well plates. The migration assay plates were placed in a humidified incubator (37 °C, 5% CO_2) for 4 hours. After 4 hours, the transwells were removed, medium and cells from the lower wells were harvested, and the wells were rinsed once with 0.5 ml MACS buffer (PS00000001). Harvested cells with rinses were pelleted by centrifugation; cells were then resuspended in a small volume of MACS buffer (50-100 µl) and left on ice until counted. The numbers of viable cells present per sample were determined by counting cells able to exclude trypan blue.

Cell Culture and RNA Isolation for DNA Microarray Experiments

For analyses of gene expression, B cells were cultured for varying periods of time in SIMDM (PS00000056) -/+ 10% FBS (Hyclone, cat. No. SH30070.03) in 6-well Costar ultralow plates (Fisher Scientific, cat. no 07-200-601) at 6 x 10^6 cell/ml, 3 ml per well. For ligand stimulation, cells were harvested from the 6-well plates, counted for viability, and replated at 18 x 10^6/well in 12-well Costar plates. Cells were rested for 1 hour prior to ligand stimulation with a final volume after ligand addition of 1.2 ml/well, cell density at 16.7 x 10^6/ml. Cells were treated with ligands for specified times and then harvested for RNA isolation (PP00000009). RNA quality was determined by running samples out on agarose gels (PP00000025). Microarray experiments used transcript profiling analyses to examine relative gene expression in paired samples (PP00000019), as described in the text (see sections: Gene Expression Changes in Response to In Vitro Culture and Anti-IgM Stimulation, above, and DNA Microarray Data Analyses, below).

DNA Microarray Data Analyses

In the analysis of Agilent array microchips, which were probed with competing red and green labeled probes derived from the original RNA samples, features with a saturated, below background, or less than 300 pixels green or red intensity in a given array were removed. For a given hierarchical clustering analysis, only features that had at least 80% nonblank measurements and an average of twofold or more changes in at least one experimental condition were included. Hierarchical clustering was done with the Multiple Array Viewer developed by The Institute of Genetic Research (TIGR). Euclidian distance was used as the similarity metric. The statistical significance of coclustering of genes involved in the same biological function/process was computed with CLASSIFI analysis (http://pathcuric1.swmed.edu/pathdb/classifi.html).

Lentivirus Production and Transduction

We used a combination of three plasmids, including a vector plasmid, pFUGW (14), with the pCMVÄR8.91 packaging plasmid (31, 34), and the pMD.G envelope plasmid (31, 34), to produce nonreplicating HIV-based lentivirus following transfection of 293T packaging cells. The vector plasmid contained viral RNA packaging signal sequence and GFP marker protein between self-inactivating viral long terminal repeats (SIN-LTRs). The three LTRs were altered by deletion to become SIN-LTRs that are self-inactivated upon reverse transcription and integration after infection of the target host cell. pFUGW came from the laboratory of David Baltimore, and in this plasmid eGFP is expressed under the ubiquitin C promoter between viral SIN-LTRs. The pCMVÄR8.91 'packaging' plasmid (Trono lab, Geneva) expresses HIV gag, pol, and rev genes, but does not carry viral RNA packaging sequence, so these genes do not get carried in the nonreplicating lentivirus that is produced. The envelope plasmid, pMD.G (Trono lab, Geneva) expresses vesicular stomatitis virus (VSV) G envelope protein, which binds cell membrane lipids and, hence, permits entry of the pseudotyped lentivirus produced into all mammalian cells. 293T packaging cells were transfected with three lentiviral plasmid combinations using the Lipofectamine 2000 (Gibco-BRL, Life Technologies, Inc.) following AfCS protocol PP00000200. The lentivirus preparations produced were used with and without concentration (PP00000202), and infections of either stimulated or nonstimulated B cells and the Ba/F3 pro-B cell line were performed in the presence of polybrene (Sigma-Aldrich, cat. no. P1274 at 4 µg/ml), according to AfCS protocol PP00000215.

References

1. Sharp PA. (2001) Genes Dev. 15(5), 485-490.
2. Hannon GJ. (2002) Nature 418(6894), 244-251.
3. Dillin A. (2003) Proc. Natl. Acad. Sci. U.S.A. 100(11), 6289-6291.
4. Bennet CF and Cowsert LM. (1999) Biochim Biophys Acta. 1489(1), 19-30.
5. Strasser A, Harris AW, Vaux DL, et al. (1990) Curr. Top. Microbiol. Immunol. 166, 175-181.
6. McDonnell TJ, Deane N, Platt FM, et al. (1989) Cell 57(1), 79-88.
7. Strasser A, Whittingham S, Vaux DL, et al. (1991) Proc. Natl. Acad. Sci. U.S.A. 88(19), 8661-8665.
8. Merino R, Ding L, Veis DJ, Korsmeyer SJ, and Nunez G. (1994) EMBO J. 13(3), 683-691.
9. Cory S and Adams JM. (2002) Nat. Rev. Cancer 2(9), 647-656.
10. Rolink AG and Melchers F. (2002) Curr. Opin. Immunol. 14(2), 266-275.
11. Mackay F and Browning JL. (2002) Nat. Rev. Immunol. 2(7), 465-475.
12. Grewal IS and Flavell RA. (1997) Immunol. Res. 16(1), 59-70.
13. Grewal IS and Flavell RA. (1998) Annu. Rev. Immunol. 16, 111-135.

14. Qin XF, An DS, Chen IS, and Baltimore D. (2003) Proc. Natl. Acad. Sci. U.S.A. 100(1), 183-188.
15. Rubinson DA, Dillon CP, Kwiatkowski AV, et al. (2003) Nat. Gen. 33(3), 401-406.
16. Hardy RR and Hayakawa K. (2001) Annu. Rev. Immunol. 19, 595-621.
17. Brunner C, Marinkovic D, Klein J, Samardzic T, Nitschke L, and Wirth T. (2003) J. Exp. Med. 197(9), 1205-1211.
18. Lam KP, Kuhn R, and Rajewsky K. (1997) Cell 90(6), 1073-1083.
19. O'Reilly LA, Harris AW, Tarlinton DM, Corcoran LM, and Strasser A. (1997) J. Immunol. 159(5), 2301-2311.
20. Plas DR and Thompson CB. (2002) Trends Endocrinol. Metab. 13(2), 75-78.
21. Downward J (2003) Nature 424(6951), 896-897.
22. Plas DR, Rathmell JC, and Thompson CB (2002) Nat. Immunol. 3(6), 515-521.
23. Danial NN, Gramm CF, Scorrano L, et al. (2003) Nature 424(6951), 952-956.
24. Satterthwaite AB, Willis F, Kanchanastit P, et al. (2000) Proc. Natl. Acad. Sci. U.S.A. 97(12), 6687-6692.
25. Niiro H and Clark EA. (2002) Nat. Rev. Immunol. 2(12), 945-956.
26. Fruman DA, Ferl GZ, An SS, Donahue AC, Satterthwaite AB, and Witte ON. (2002) Proc. Natl. Acad. Sci. U.S.A. 99(1), 359-364.
27. Reif K, Ekland EH, Ohl L, et al. (2002) Nature 416(6878), 94-99.
28. Lois C, Refaeli Y, Qin XF, and Van Parijs L. (2001) Curr. Opin. Immunol. 13(4), 496-504.
29. Naldini L, Blomer U, Gallay P, et al. (1996) Science 272(5259), 263-267.
30. Zufferey R, Dull T, Mandel RJ, et al. (1998) J. Virol. 72(12), 9873-9880.
31. Salmon P, Kindler V, Ducrey O, Chapuis B, Zubler RH, and Trono D. (2000) Blood 96(10), 3392-3398.
32. Chinnasamy D, Chinnasamy N, Enriquez MJ, et al. (2000) Blood 96(4), 1309-1316.
33. Bovia F, Salmon P, Matthes T, et al. (2003) Blood 101(5), 1727-1733.
34. Rossi GR, Mautino MR, and Morgan RA. (2003) Hum. Gene. Ther. 14(4), 385-391.

Authors*

Tamara I. A. Roach[†‡]
Sangdun Choi[§]
Heping Han[‖]
Xiaocui Zhu[§]

Acknowledgements

Technical Assistance

Eduardo Arteaga[‖]
Mi Sook Chang[§]
Melissa Kachura[‡]
David Quan[‡]
Carrie Wong[‡]
Nicholas Wong[‖]

* Please refer to the AfCS policy on authorship.
† To whom scientific correspondence should be addressed.
‡ Assay Development Laboratory, San Francisco Veterans Administration Medical Center, San Francisco, CA.
§ Molecular Biology Laboratory, California Institute of Technology, Pasadena, CA.
‖ Antibody Laboratory, University of Texas Southwestern Medical Center, Dallas, TX.
¶ To whom questions or comments about the AfCS Research Reports should be addressed.