

Purification and Characterization of Mouse Splenic B Lymphocytes

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Abstract

Initial efforts of Alliance for Cellular Signaling (AfCS) laboratories have focused on establishing standard and reproducible procedures for purifying B lymphocytes (B cells) from mouse spleen. Our goal was to obtain cultures that permit reliable measurement of short-term signaling events and changes in gene expression. This report describes a negative-selection procedure for B-cell enrichment and characterization of the isolated cell population using multiparameter fluorescence-activated cell sorting (FACS) analysis. Results show that we obtain approximately 47×10^6 cells per spleen, of which 96% contain the B220 marker typically expressed on B cells. More than 80% of these cells can be further characterized as resting, mature B2 cells. Conditions for maintaining viable cells in serum-free medium for short-term culture were defined. Supplemented Iscove's Modified Dulbecco's Medium (SIMDM) was most consistent among several media in maintaining cell viability for 24 hours. B cells also showed consistent and reproducible signaling responses to various stimuli. The cell preparations contain few non-B-cell contaminants and exhibit expected heterogeneity in the expression of some cell-surface markers. The procedure described is conveniently transferable and provides a suitable model for the study of global signaling responses in primary cells.

Introduction

The overall goal of the Alliance for Cellular Signaling (AfCS) is to understand, as completely as possible, how cells interpret signals in a context-dependent manner. How do cells respond appropriately to individual signals while they are bombarded with many simultaneously? How do various cellular signaling modules interact to form a robust control system? The pathway to such understanding will involve identifying all the proteins that compose the various signaling systems, constructing a physical map of their relationships, assessing time-dependent information flow through the pathways, and, finally, reducing the mass of detailed data into a set of interacting theoretical models that describe cellular signaling.

One premise of the AfCS is that we must focus intense effort on a small number of cellular signaling systems in order to gather the requisite amount of data. An extensive [list of criteria](#) was evaluated to aid in these choices. Particularly important among these were the capacity to observe interesting regulated phenotypes, to obtain relatively large numbers of homogeneous cells, and

to manipulate gene expression in these cells based on reasonably complete knowledge of their genome. We also wish to study relatively normal cells from a mammalian organism. Based on these criteria one of the cells chosen was the resting B lymphocyte (B cell) of the mouse.

By secreting antigen-specific immunoglobulin antibody, the B cell plays an essential role in host defense. Resting B cells reside in the circulation and migrate to the spleen and lymph nodes. Activation of the B-cell receptor by antigen in conjunction with appropriate co-stimulation causes the resting B cell to proliferate and differentiate into a plasma cell, which makes and secretes large amounts of immunoglobulin antibody. In addition to the antigen receptor, B cells exhibit a large number of additional cell surface receptors, which are activated by T-cell surface molecules, cytokines, bacterial endotoxin, and other ligands. Immunologists have begun to define key steps in signaling pathways downstream of these receptors that regulate steps in development of the mature B cell and modulate responses to antigen (1,2,3).

The best-studied G protein-coupled receptors on B cells activate G_i and mediate directional motility in response to chemokines. This chemotactic response, which is essential for homing of B cells to their proper location in the spleen and lymph nodes (4), offers a fitting challenge for the AfCS. Chemotactic signals within migrating cells are just beginning to be identified but already include a vast array of different signaling families, including heterotrimeric G proteins; rho GTPases and their exchange factors, GTPase activating proteins, and effectors (e.g. kinases); tyrosine kinases; lipid kinases; and protein kinases A, B, and C (5,6). The challenge is not only that we know little about the wiring that connects these signaling elements, but even more importantly, we must identify the underlying compass mechanism—how the signaling machine interprets chemotactic gradients in space and time—to decide where the cell should go.

To approach this attractive and challenging cellular system, the AfCS must develop and apply standardized, reproducible procedures for isolating and purifying resting splenic B cells and for their short-term maintenance in culture under defined conditions. These procedures must also be conveniently transferable between laboratories, not only within the AfCS, but also to laboratories of non-AfCS investigators. This report describes the procedures that we have adopted to meet these criteria and documents the utility of the isolated cells for short-term (hours) studies of their signaling mechanisms.

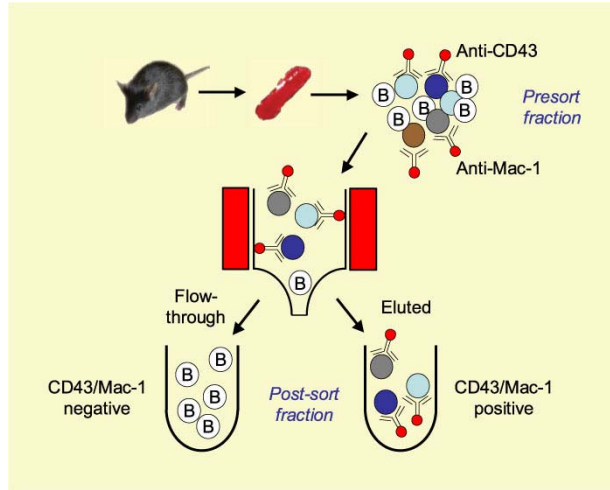


Fig. 1. Schematic representation of B-cell purification by negative selection. Spleens are removed from anesthetized mice and dispersed through a nylon mesh to generate a single-cell suspension. The splenocytes are washed (following removal of erythrocytes by osmotic shock) and incubated with anti-CD43 and anti-Mac-1 antibody-conjugated microbeads (Miltenyi Biotec). The bead-bound cells (positive fraction) are separated from unbound cells (negative fraction) using an AutoMACS magnetic cell sorter. The magnetized column retains the positive fraction while the negative fraction containing resting B cells is collected in the flow-through. The positive fraction is later eluted for examination.

Results

Isolation of Splenic B Cells

A negative-selection procedure developed by Miltenyi and colleagues (7) was chosen for purification of splenic B cells (Fig. 1). This method relies on the fact that most leukocytes express CD43; resting mature B cells are exceptions. Expression of CD43 has been demonstrated on immature B cells, plasma cells, and some mature B1 cells, in addition to granulocytes, monocytes, macrophages, platelets, natural killer (NK) cells, thymocytes, and peripheral CD8^{pos} and most CD4^{pos} T cells (8, 9, 10, 11, 12). The heterogeneous cell population isolated from spleen was incubated with paramagnetic microbeads coupled to an anti-CD43 monoclonal antibody. To improve the removal of non-B cells (specifically myeloid contaminants), anti-Mac-1 (CD11b) beads were also included during the negative-selection procedure. The beads with unwanted cells attached were then removed using an AutoMACS computer-controlled magnetic cell sorter (Miltenyi Biotec, Auburn, CA). Resting, mature B cells remain relatively unperturbed during this procedure, and cell activation associated with positive selection is avoided.

The enriched cell population was subjected to multiparameter fluorescence-activated cell sorting (FACS) analysis to estimate the percentage of cells expressing B220, a marker present on cells committed to the B lineage

(Fig. 2) (13, 14, 15). Contaminating cell populations that remained after enrichment were examined by staining the leukocyte markers Mac-1, CD43, Gr-1, and CD3 (Fig. 2). The enriched cell fraction contained approximately 47×10^6 cells/spleen, of which 96% expressed B220 (B220^{pos}) (Table 1). Only small numbers of CD43^{pos}/Mac-1^{pos} cells were present. Blockade of the Fc receptor on splenocytes prior to incubation with magnetic beads did not improve yields significantly (not shown). Further characterization of the isolated cells is described below (see *Cell Characterization*).

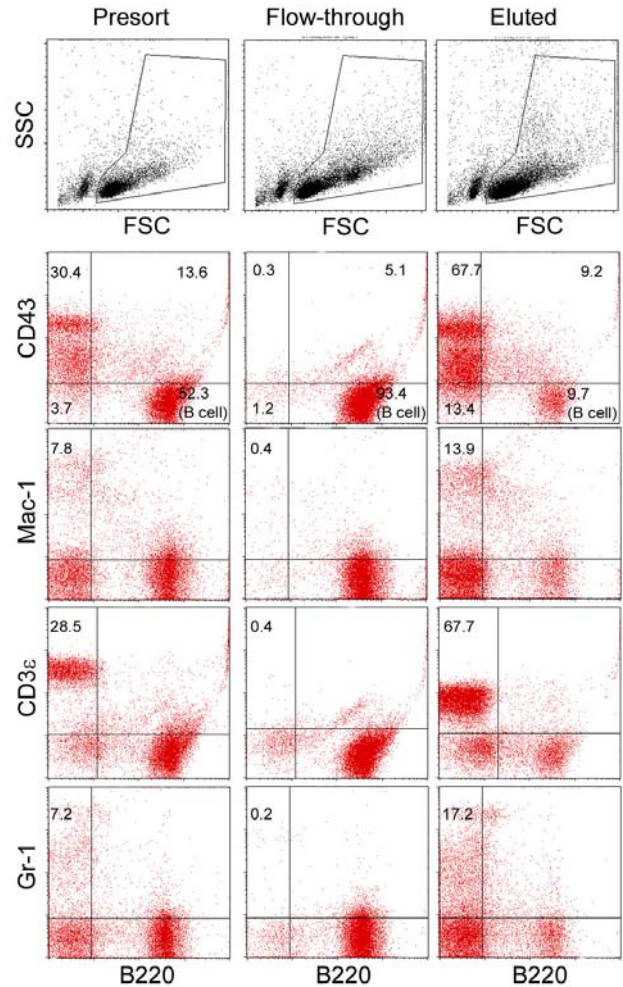


Fig. 2. Composition of the cell pools before and after sorting. The effectiveness of the negative selection was evaluated by four-color FACS analysis using antibodies to CD43, Mac-1, CD3ε, Gr-1, and B220. Cells were examined by forward scatter (FSC) and side scatter (SSC) to identify the viable cell fraction (first row). Each plot represents a typical analysis from 20 experiments; the percentage of cells expressing Mac-1 (macrophages), CD3ε (T cells), Gr-1 (granulocytes), or B220 (B cells) is shown in the appropriate quadrants. Positioning of the cross-hair gates was done on control plots derived from cells stained only for B220.

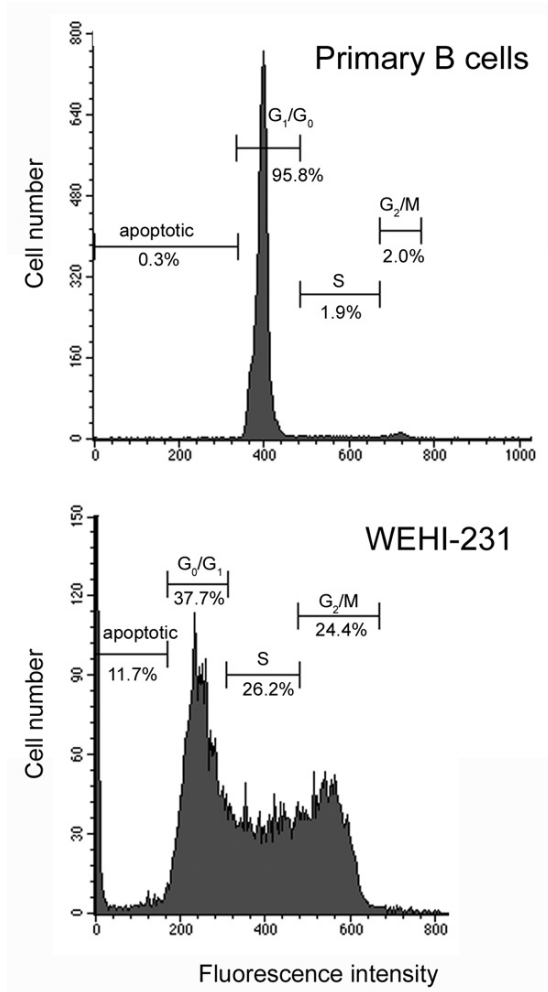


Fig. 3. Cell cycle distribution of isolated splenic B cells and a cultured, mouse B-cell lymphoma, WEHI-231. Freshly isolated splenic B cells and WEHI-231 cells were stained with 1 µg/µl of propidium iodide and examined by FACS. The distribution of cells throughout the cell cycle was estimated from their total DNA content (measured by relative fluorescence intensity). Representative histograms are shown.

To evaluate the proliferative status of the isolated B cells, cellular DNA content was determined by FACS analysis after staining with propidium iodide (Fig. 3). More than 95% of the cells resided within the G₀/G₁ phases of the cell cycle. Propidium iodide staining of a proliferating B lymphoma cell line, WEHI-231, is shown for comparison; only 38% of these cells were in G₀/G₁ phases of the cell cycle.

The Laboratory for Development of Signaling Assays (Veterans Administration Medical Center, San Francisco) and the Cell Preparation and Analysis Laboratory (University of Texas Southwestern Medical Center, Dallas) both isolate and culture B cells for AfCS experiments. It is essential that we are able to replicate these procedures at these sites. This will ensure that others in the research community can reproduce and extend our

observations. The data presented in Table 1 demonstrate that the cell populations isolated in our two laboratories appear very similar. However, results from the Cell Preparation and Analysis Laboratory demonstrate a consistent trend of obtaining a greater number of starting cells per spleen and consequently greater yields of B220^{pos} cells. We cannot currently offer a clear explanation for this difference.

Cell Characterization

Characterization of specific subpopulations, developmental stage, and activation status of isolated splenic B cells was performed by multiparameter FACS analysis. The analysis described here uses specific antibodies coupled to one of four fluorochromes: fluorescein isothiocyanate (FITC), R-phycoerythrin (PE), peridinin chlorophyll-a (PcP), or allophycocyanin (APC). These fluorochromes were used concurrently to assess the cell-surface expression of four different markers within the same sample. Isotype-matched antibodies against keyhole limpet hemocyanin (KLH) were used as controls.

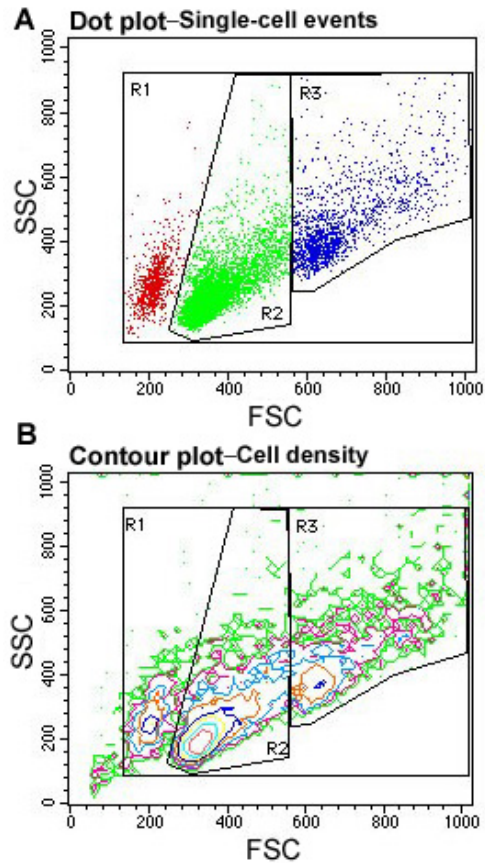


Fig. 4. Size and uniformity of splenic B cells. B cells profiled using forward scatter (FSC) and side scatter (SSC), viewed on dot plot (A) or contour plot (B). Dead cells falling in the bottom left corner of region 1 (R1), are colored red. R2 and R3 are drawn within R1, marking smaller less granular cells (R2=green) and larger more granular cells (R3=blue).

Table 1. Summary of primary B-cell preparations. Cells were counted with a hemocytometer under a phase-contrast microscope. Viability was determined by trypan blue exclusion. B220^{pos} cells were quantified by FACS analysis. Data are mean ± SD. The number in parentheses is the number of experiments in which the information was quantified. Calculated values are the average of specified determinations. ND=Not determined.

	Cell Preparation and Analysis Lab [†]		Assay Development Lab	
Body Weight (g)	ND		25.8 ± 2.2	(190)
Spleen Weight (mg)	ND		84.1 ± 12.8	(192)
Spleen Weight/Body Weight (x10 ³)	ND		3.3 ± 0.5	(190)
Presort				
Viable (%)	81.9 ± 3.6	(19)	79.2 ± 5.4	(19)
Cells/Spleen (x10 ⁶)	112.0 ± 14.0	(19)	98.7 ± 16.0	(20)
B220 ^{pos} (%)	56.1 ± 4.5	(19)	62.2 ± 5.9	(16)
B220 ^{pos} Cells/Spleen (x10 ⁶)	62.5 ± 9.2	(19)	63.3 ± 13.9	(16)
Post-Sort Flow-Through (B-Cell)				
Viable (%)	90.4 ± 4.0	(19)	95.1 ± 1.9	(18)
Cells/Spleen (x10 ⁶)	53.7 ± 8.2	(19)	41.4 ± 6.6	(20)
B220 ^{pos} (%)	96.2 ± 1.6	(19)	96.3 ± 2.7	(20)
B220 ^{pos} Cells/Spleen (x10 ⁶)	52.2 ± 7.7	(19)	39.8 ± 6.4	(20)
Yield (%)*	82.8 ± 8.2	(19)	65.4 ± 16.8	(18)
Post-Sort Eluted				
Viable	87.7 ± 5.5	(19)	87.7 ± 4.5	(7)
Cells/Spleen (x10 ⁶)	36.7 ± 11.2	(19)	29.7 ± 5.9	(8)
B220 ^{pos} (%)	15.0 ± 3.0	(19)	26.6 ± 1.7	(17)
B220 ^{pos} Cells/Spleen (x10 ⁶)	5.5 ± 2.0	(19)	7.5 ± 2.3	(8)

*Yield = (number of B220^{pos} cells in the flow-through)/(number of B220^{pos} cells in the total pool). [†] All preparations were from 16 mice each.

Purity. The purity of the B cells obtained from mixed splenocyte populations was assessed in part by expression of the B220 isoform of CD45 (leukocyte common antigen, Ly-5, or T200) (13, 14, 15). Although B cells are the predominant leukocyte expressing B220, the marker is also displayed by subsets of NK cells, activated T cells, and a subpopulation of dendritic cells (16). Hence, the purity of our preparations cannot be assessed by expression of B220 alone. The absence of T-cell and myeloid-cell markers, in addition to the concomitant expression of CD23 and membrane-bound IgD and IgM, were also used to evaluate heterogeneity within the cell population.

Although the vast majority of negatively selected cells are characterized as B220^{pos}, CD3^{neg}, Mac-1^{neg}, and IgD^{pos} (consistent with mature resting B cells), the enriched B cells are not a homogeneous population (Figs. 2 and 5). Forward scatter (FSC) and side scatter (SSC) analysis of the selected B cells consistently showed a subpopulation of relatively larger cells, marked as region 3 (R3) (Fig. 4), that carry the same markers as the smaller cells in region 2 (R2). These larger cells may represent cell pairs. If so, pairs are maintained in the presence of 0.5 mM EDTA, and these cellular interactions may be particularly tight. These cells typically constitute less than 15% of the negative fraction. Cells in the bottom left corner of region 1 (R1) (Fig. 4) are dead.

B cells are classified into two major groups based on their ontogeny (17). B2 cells are bone marrow-derived and make up the bulk of splenic B cells. B2 cells characteristically have high levels of B220, membrane-

bound IgD, and CD23 (IgE Fc Receptor, FcεR). Conversely, B1 cells arise from precursors present during fetal development and reside predominantly in the pleural and peritoneal cavities of the adult mouse. B1 cells usually display low-to-intermediate amounts of B220, no or intermediate amounts of CD23, and no or low levels of IgD. B1 cells share some of these characteristics with marginal zone (MZ) B cells. Hence, examination of the levels of B220 and CD23 allow the B2 cells to be distinguished from B1, MZ, and transitional immature B cells (T1 or T2, newly arrived in the spleen). Analysis of the B220^{pos} fraction suggests that roughly 80% to 85% of the selected B cells are of the B2 type, as defined by expression of IgD (Fig. 5E) and CD23 (Fig. 6B,D).

Splenic MZ B cells, like B1 cells, are a noncirculating population of B cells distinct from the predominant B2 follicular (FO) population (18). MZ B cells express high levels of membrane-bound IgM and the complement receptors, CR2 and CR1 (CD21 and CD35), while having little or no CD23. MZ B cells normally compose only a small fraction of the splenic B-cell population, as shown in Fig. 6A and C and B1 cells constitute even a smaller fraction. (Note that the monoclonal antibody used to stain CD21 recognizes an epitope shared by CR2 and CR1).

Analysis of 23 distinct B-cell preparations is shown in Supplemental Table A (available online). Purity was determined as described above. The data show that we consistently isolate splenic B cells that are primarily (>80%) composed of mature B2 cells.

Activation State. The expression of common lymphocyte activation markers was also examined by flow cytometry. Freshly isolated cells were stained for B220 and CD69, CD25, CD80, CD86, CD30, or Ly-77. Fig. 7 shows histograms of cells stained with each marker compared to a corresponding isotype-matched antibody control. B220^{pos}-gated cells show low levels of CD69 (very early antigen) and CD25 (α chain of the IL-2 receptor), both of which can be up-regulated on T and B cells after activation (19, 20). Similarly, CD80, a coreceptor induced on B cells and necessary for T-cell activation (21), is expressed at low levels in our B-cell population. CD86 (B7-2) and CD30 were not detected.

Interestingly, Ly-77 is expressed heterogeneously within this cell population. Taken together with the low percentage of cells observed within the S, G2, and M phases of the cell cycle (Fig. 3), these data indicate that the cells are in a resting state.

We also evaluated expression of adhesion markers commonly found in B cells (Fig. 7). Within the isolated B220^{pos} B-cell fraction, CD29 (integrin β 1 chain) is expressed at a low level by some cells. CD62L (L-selectin) is highly expressed with a broad distribution, and CD54 (LFA-1) is expressed rather homogeneously. This again demonstrates some heterogeneity within the isolated B-cell population.

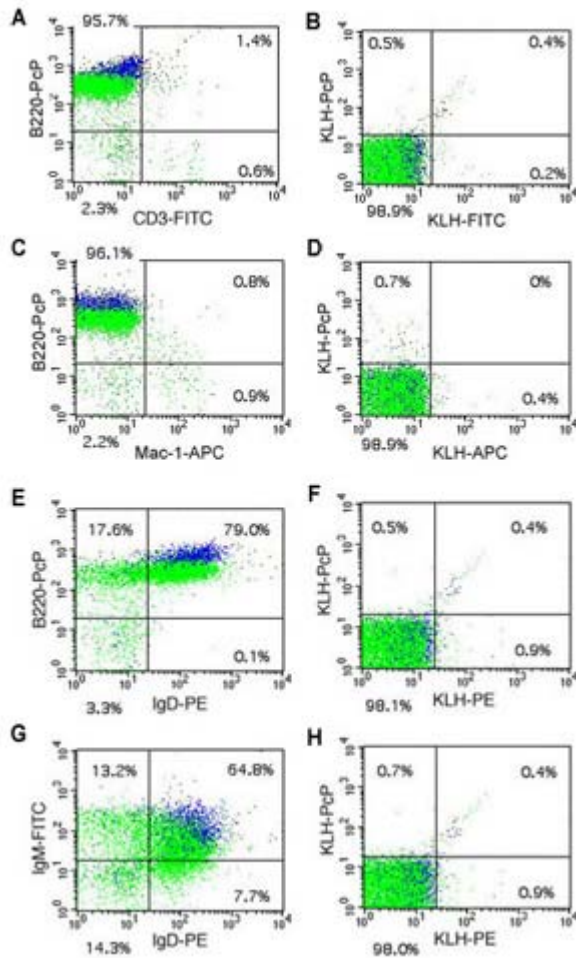


Fig. 5. Purity of B-cell preparations. Live cells (R2 and R3) were examined. Cells stained positively for B220 but not (A) CD3 (T-cell marker) or (C) Mac-1 (myeloid cell marker). (E) The majority (~81%) of B220 positive cells also stained positively for membrane IgD, which is present on mature B cells. (G) Most cells (64.8%) stained for both IgD and IgM, although some were positive only for IgD (13.2%) or IgM (7.7%). Plots of cells stained with isotype-matched control antibodies against keyhole limpet hemocyanin (KLH) coupled to FITC, PE, PcP or APC (B,D,F,H) are shown to the right of its corresponding test pair (A,C,E,G).

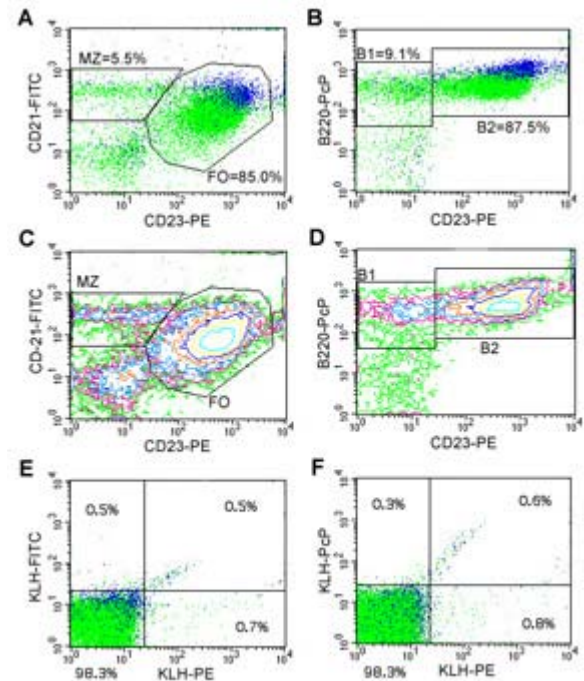


Fig. 6. Heterogeneity of B cells. Live cells (R2 and R3) were examined. (A,C) B-cell subpopulations were identified by anti-CD21-FITC versus CD23-PE staining. Follicular B cells express high levels of CD23 and CD21 and are marked in the FO region. Marginal zone B cells express high levels of CD21, but do not express CD23, and are marked in the MZ region. (B,D) B-cell subpopulations were identified by anti-B220-PcP and anti-CD23-PE staining. B2 cells express B220 and high levels of CD23. B1 cells express B220, but have little or no CD23. (E,F) Cells stained with isotype-matched antibodies against keyhole limpet hemocyanin (KLH) coupled to FITC, PE or PcP.

Gene Expression. A profile of gene expression in isolated splenic B cells was obtained in a collaborative effort with the Genomics Institute of the Novartis Research Foundation (GNF)(22). Two independent experiments were performed using total RNA purified from separate B-cell preparations. Expression was examined with mouse Affymetrix GeneChip arrays (MGU74Av2) using a standard protocol provided by Affymetrix. Average difference values that estimate transcript expression levels

were calculated, and judgments were made as to the presence or absence of each transcript. Equivocal results were termed *marginal*. Data can be viewed in the [Supplemental Data](#) file (available online).

Of 12,488 probe sets screened, 3,664 (29%) yielded positive results in both hybridization experiments. As expected, many probe sets with high expression values represent genes normally expressed in B cells. For instance, transcripts for CD45, CD22, CD23, CD37, CXCR4, CXCR5, and B-cell receptor components (e.g., heavy chain of IgM) were present, whereas transcripts for proteins typically expressed in T cells such as CD3 and CD8, were absent. Many transcripts that are restricted to the myeloid lineage were also absent, although expression of CD14 and CD68 was suggested. Several transcripts typically restricted to erythroid cells (e.g., kruppel-like factor 1, hemoglobin alpha and beta chains) were also present. The data are consistent with the characterization performed by FACS and suggest the presence of few myeloid and erythroid cells in our preparations.

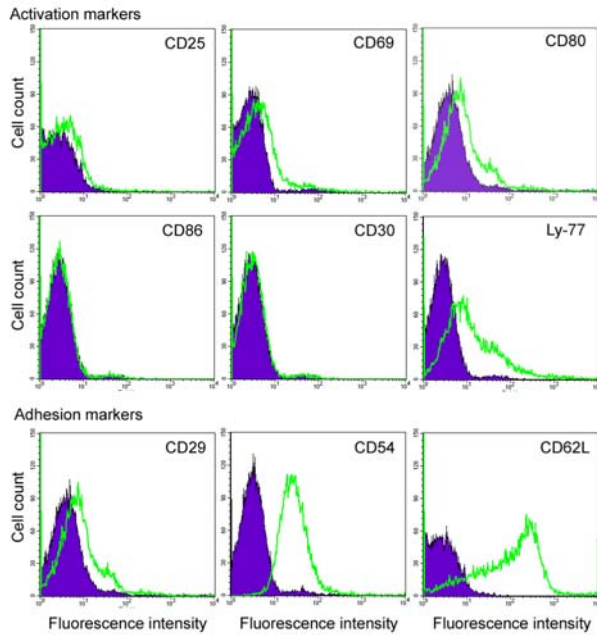


Fig. 7. Expression of activation and adhesion markers on isolated B cells. Expression of CD25, CD69, CD80, CD86, CD30, Ly-77, CD29, CD54, and CD62L was evaluated on B cells by four-color flow cytometry using fluorochrome-conjugated antibodies. All samples were gated on B220. Each histogram is representative of four experiments. The filled histograms represent cells stained with an isotype control antibody. The open histograms represent staining for the specific surface marker.

Short-Term Cell Culture

We sought to identify culture conditions to meet several requirements. B cells should be maintained under conditions that 1) are standardized and reproducible between cell preparations; 2) minimally perturb their

internal regulatory circuits; and 3) are convenient and easily transferable between AfCS and other laboratories. Thus, B cells should be cultured in a defined medium, without the use of serum or similar additives. We relied primarily on measures of B-cell viability to select such a medium. We also examined other variables, including functional integrity and recovery of B cells during experimental manipulations.

We tried to devise culture conditions that would be suitable for most assays and manipulations of splenic B cells. At the same time, we recognize that this will not always be possible; for instance, the presence of albumin promotes consistent availability of ligands to cells in medium (e.g., prevents trapping in plastic), but it may cause problems for short-term experiments better performed in the absence of added proteins (e.g., gel electrophoresis). We describe here the medium chosen for most culture conditions and assays.

Selection of a Base Medium. B-cell viability was measured in several commercial media including Iscove's Modified Dulbecco's Medium (IMDM), which was originally developed to support primary B lymphocytes in vitro (23). Other media tested included an Iscove's-based medium developed by Mosier (24), which contains a 1:1 mixture of IMDM and Ham's F-12 (Mosier Modified Medium, MMM); Dulbecco's Modified Eagle's Medium (DMEM); RPMI 1640, a common medium for immune cell culture; and Medium 199 (M199). [Supplemental Table B](#) (available online) lists the formulations for each of these media. All media contain 12.5 mM HEPES except IMDM, which contains 25 mM HEPES; except as noted, all media were initially supplemented with insulin (10 μ g/ml), transferrin (5.5 μ g/ml), and selenium (5 ng/ml) (ITS medium supplement), L-glutamine (2 mM), β -mercaptoethanol (55 μ M), penicillin (100 U/ml), and streptomycin (100 μ g/ml). Additives other than β -mercaptoethanol were added to 500 ml of medium, which was which kept refrigerated for up to a week. β -mercaptoethanol was added immediately prior to use.

B-cell viability was first assessed at 0 and 24 hours using the Live/Dead Assay ([Molecular Probes](#)). At 24 hours, B-cell viability in IMDM ($65.5 \pm 1.8\%$) and in MMM ($59.8 \pm 0.9\%$) was clearly better than in DMEM, RPMI, or M199 ([Fig. 8A](#)). IMDM and MMM were thus further tested for their capacity to maintain cell viability at 4 hours, since AfCS experiments will initially focus on short-term responses to ligands for various receptors. At 4 hours, B-cell viability was $86.5 \pm 2.4\%$ in IMDM and $77.4 \pm 6.0\%$ in MMM ([Fig. 8B](#)).

Several commercial media are designed specifically for cell culture in the absence of serum. We compared IMDM with two such formulations, [GIBCO Hybridoma Serum-Free Medium \(HSFM\)](#) and [GIBCO CD medium](#), by assessing B-cell viability at 24 hours. B-cell viability was similar in IMDM and HSFM and only

slightly higher in CD medium (Fig. 8D). Although HSFM and CD media are defined and readily available, their compositions are proprietary. Since IMDM proved similar in its capacity to support B-cell viability, we chose to continue using this nonproprietary formulation.

Effect of Cell Density. B cells were cultured in both IMDM and MMM at densities ranging from 1.25 to 20 x10⁶ cells/ml, and viability was assessed at 24 hours. B-cell viability was greatest and most consistent at all densities in IMDM, whereas viability increased progressively with increasing density in MMM, eventually equaling that observed in IMDM (Fig. 8C).

Effect of Additives to IMDM. B-cell viability at 4 or 24 hours was not affected by removal of insulin,

transferrin, or the ITS medium supplement (Table 2). Addition of bovine serum albumin (BSA, specifically shown to lack bacterial lipopolysaccharide) was tested at concentrations of 0.005 to 0.1 mg/ml with no effect on viability (data not shown). Other additives including streptomycin, penicillin, dextran sulfate, polyethylene glycol (PEG), and cholesterol, had no effect on B-cell viability at 24 hours. Other additives were also tested in MMM and were similarly without effect. These included stearate, ethanolamine, Tween 80 (a lipid formulation found in M199), and taurine. Pluronic F-68 was tested at concentrations ranging from 0.0125% to 0.1%; this improved cell recovery during centrifugation when used at a concentration of 0.025% with no effect on viability.

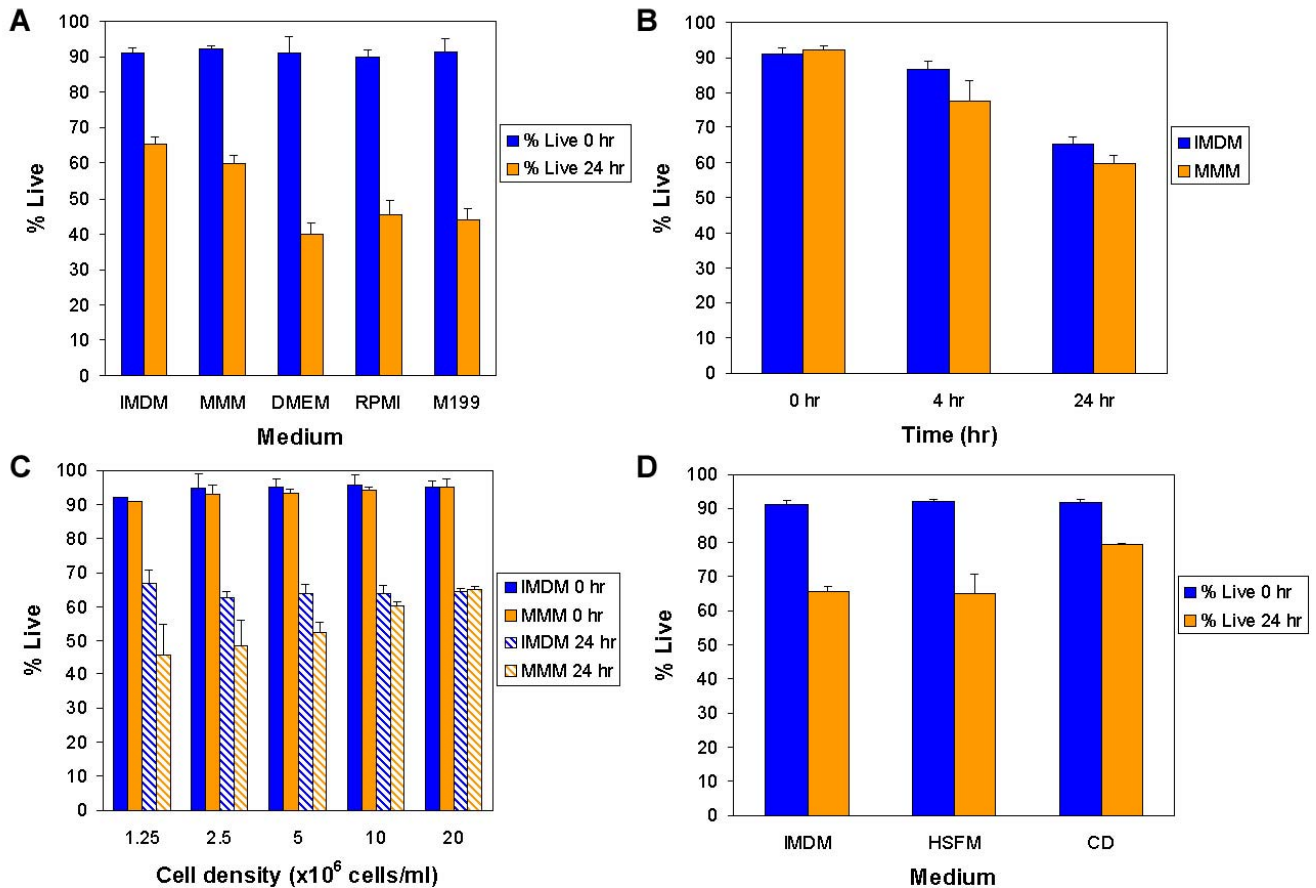


Fig. 8. Viability of B cells cultured in defined media. Viability was measured using the Live/Dead Assay (Molecular Probes) and is shown as the percentage of live cells present at indicated times (calculated as the number of live cells at time x divided by the number of total cells at time x). Data are the mean ± range of “n” experiments. **(A)** B Cells were cultured in either (i) Iscove’s Modified Dulbecco’s Medium (IMDM) containing 25 mM Hepes; (ii) Mosier Modified Medium (MMM), which is 1:1 IMDM:Ham’s F-12 medium (12.5 mM Hepes); (iii) Dulbecco’s Modified Eagle’s Medium (DMEM) with Earle’s Balanced Salt Solution; (iv) RPMI 1640 (RPMI); or (v) Medium 199 (M199). In these experiments, all media were supplemented with insulin (10 µg/ml), transferrin (5.5 µg/ml), and selenium (5 ng/ml) (ITS Medium Supplement, Sigma), 2 mM L-glutamine, 55 µM β-mercaptoethanol, 100 U/ml penicillin, and 100 µg/ml streptomycin. In addition, DMEM, RPMI, and M199 were supplemented with 12.5 mM Hepes (GIBCO). Viability was measured at 0 and 24 hr (IMDM n = 14, MMM n = 15, DMEM, RPMI, and M199 n = 3). **(B)** Cells were cultured in IMDM or MMM and viability was determined at 0, 4, and 24 hr (IMDM 4 hr n = 4, 24 hr n = 16; MMM 4 hr n = 6, 24 hr, n = 16). **(C)** B cells were cultured in IMDM or MMM at cell densities ranging from 1.25 to 20 x10⁶ cells/ml and viability was determined at 24 hr (n = 2). **(D)** B Cells were cultured in IMDM, Hybridoma Serum-Free Medium (HSFM) or CD Medium (both from GIBCO). HSFM and CD media were supplemented with 2 mM L-glutamine, 55 µM β-mercaptoethanol, 100 U/ml penicillin, and 100 µg/ml streptomycin. Viability was measured at 0 and 24 hr (IMDM n = 14, HSFM n = 5, CD n = 2).

Table 2. Effects of medium supplements. B cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM, containing 25 mM Hepes) supplemented with, 2 mM L-glutamine, 55 μ M β -mercaptoethanol, 100 U/ml penicillin, and 100 μ g/ml streptomycin. Medium additives were added to the IMDM in the ranges shown in the concentration column. Except for the studies specifically directed at insulin, transferrin, and selenium, media were supplemented with insulin (10 μ g/ml), transferrin (5.5 μ g/ml), and selenium (5 ng/ml) (ITS Medium Supplement, Sigma). B cells were cultured at 5×10^6 cells/ml in 200 μ l in 96-well tissue culture plates (Ultra-Low, Costar) at 37 $^{\circ}$ C in 5% CO₂. Viability was measured at 0 and 24 hrs using the Live/Dead Assay (Molecular Probes).

Additive	Concentration	Function	Effect on Viability
Insulin	10 ng/ml - 10 μ g/ml	Regulates metabolism, promotes cell survival	No effect
Transferrin	10 ng/ml - 10 μ g/ml	Iron transporter, found in mammalian sera	No effect
ITS Medium Supplement			No effect
Insulin	10 μ g/ml	Regulates metabolism, promotes cell survival	
Transferrin	5.5 μ g/ml	Iron transporter, found in mammalian sera	
Selenium	5 ng/ml	Unknown, commonly used in serum-free media	
BSA	0.005 - 0.1 mg/ml		No effect
Penicillin/Streptomycin		Prevents bacterial growth	No effect
Dextran Sulfate (64-76 KD)	1.5 nM - 1 mM	Increases colloid osmotic pressure, negative charge	No beneficial effect
Polyethylene Glycol (3-3.7 KD)	3 nM - 1 mM	Increases colloid osmotic pressure, negative charge	No effect
Cholesterol	0.1 - 8.8 μ g/ml	Major membrane lipid	No effect
Combination			No effect
Cholesterol	8.8 μ g/ml	Major membrane lipid	
Stearate	10 ng/ml	Major lipid component in commercial media	
Pluronic F-68	0.1%	Aids in recovery of cells	

Final Medium. The formulation selected for short-term culture of splenic B cells is termed Supplemented Iscove's Modified Dulbecco's Medium (SIMDM). Supplements in SIMDM include 0.025% Pluronic F-68, 2 mM L-glutamine, 55 μ M β -mercaptoethanol, and 100 μ g/ml BSA ([Supplemental Table B](#) details the composition of base IMDM). Pluronic F-68 and BSA were included because they improved cell recovery during experimental manipulation. As previously mentioned, BSA is also important for adequate presentation of many ligands to cells.

SIMDM has an osmolality of \sim 280 mOsm/kg. Plasma osmolality in the mouse is normally in the range of 310 to 325 mOsm/kg (25). Increasing the osmolality of SIMDM by addition of NaCl reduced B-cell viability (data not shown).

Functional Responses

To assess the functional integrity of our B cells, we measured ligand-induced changes in intracellular concentrations of free Ca²⁺ and cyclic adenosine monophosphate (AMP) and phosphorylation of specific sites within a panel of signaling proteins.

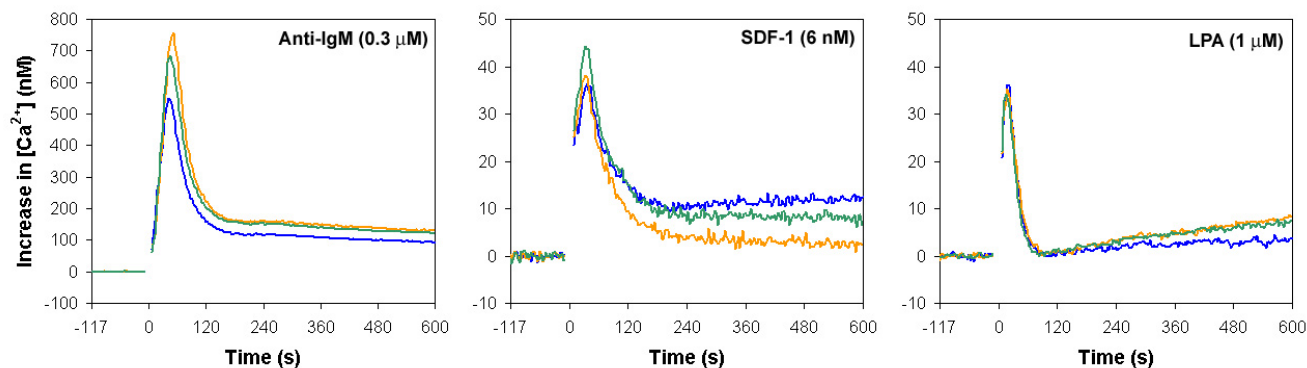


Fig. 9. Ligand-induced changes in intracellular free [Ca²⁺]. Splenic B cells were incubated with 4 μ M Fluo-3 AM at 5×10^6 cells/ml and then adjusted to 7.5×10^6 cells/ml in Hank's Balanced Salt Solution (HBSS) containing bovine serum albumin (BSA) for the assay. Anti-IgM (0.3 μ M), SDF-1 (6 nM), LPA (1 μ M), or vehicle was added at time 0. The assay was performed in a 96-well plate using a Fluoroskan Ascent Microplate Fluorometer. Measurements were recorded for 10 min followed by sequential addition of NP-40 (0.5% final) and EGTA (45 mM final) to obtain fluorescence maximum and minimum values, respectively. Intracellular free [Ca²⁺] was calculated using the equation: $[\text{free Ca}^{2+}] = K_d (F - F_{\text{min}})/(F_{\text{max}} - F)$. The K_d for Fluo-3 was assumed to be 390 nM. Data was corrected for baseline drift caused by leakage of Fluo-3 AM from cells into Ca²⁺-containing medium (see online version for details). Each graph shows tracings of three replicate samples from one experiment. Experiments were performed at least five times.

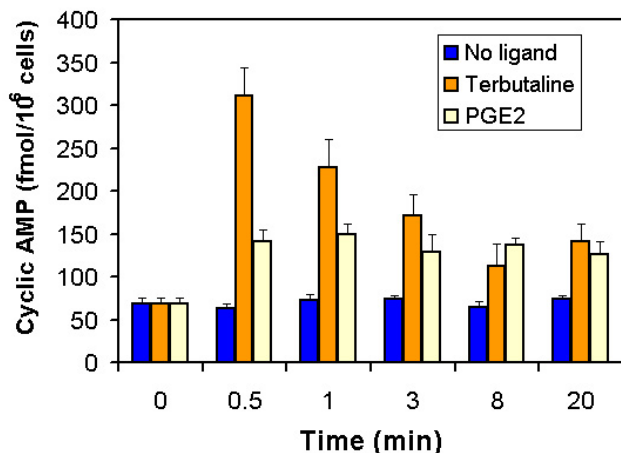


Fig. 10. **Changes in total cyclic AMP induced by terbutaline or prostaglandin E2.** B cells were incubated in 96-well plates at a concentration of 1.67×10^7 cells/ml and treated with $10 \mu\text{M}$ terbutaline, $10 \mu\text{M}$ prostaglandin E2 (PGE2), or control buffer. Lysis buffer was added directly to the wells after 0.5, 1, 3, 8, or 20 min to stop the reaction. The amount of cyclic AMP in each sample is expressed as fmol/ 10^6 cells. Each bar represents the mean \pm SEM of samples obtained from five experiments.

Ligand-Induced Changes in Intracellular Free $[\text{Ca}^{2+}]$. Ca^{2+} responses caused by polyclonal goat anti-IgM antibody ($0.3 \mu\text{M}$), SDF-1 (6 nM), or LPA ($1 \mu\text{M}$) were evaluated using the calcium-sensitive dye Fluo-3. Treatment of cells with these ligands induced a rapid and reproducible increase of intracellular $[\text{Ca}^{2+}]$ as shown in Fig 9. Three replicate tracings from one experiment are shown for each ligand. Similar tracings were obtained in additional experiments performed five or more times. Anti-IgM produced the greatest increase, which peaked at approximately 60 seconds, followed by a decrease to a plateau at approximately 20% of the peak value. SDF-1 and LPA produced smaller but similarly reproducible increases in intracellular $[\text{Ca}^{2+}]$.

Changes in Cyclic AMP Induced by Terbutaline or Prostaglandin E₂. Concentrations of cyclic AMP in B cells were determined by enzyme-linked immunosorbent assay (ELISA). Exposure of cells to the β_2 -adrenergic agonist terbutaline increases intracellular concentrations of cyclic AMP by activating the β_2 -adrenergic receptor, which in turn activates the heterotrimeric G protein G_s and the enzyme adenylyl cyclase. Prostaglandin E₂ (PGE₂) similarly activates the EP₂ and EP₄ receptors to increase cyclic-AMP concentrations. Effects of terbutaline and PGE₂ on cyclic AMP in isolated B cells were determined after exposure for 0.5, 1, 3, 8, and 20 minutes and in the absence of a cyclic nucleotide phosphodiesterase inhibitor (Fig. 10). When cells were treated with terbutaline, a rapid, transient increase in cyclic AMP was observed that peaked at 0.5 minutes, followed by a sharp decrease to a sustained level approximately 40% of the peak response. Conversely, when cells were stimulated with PGE₂, a rapid

and relatively sustained increase in cyclic AMP was observed over the 20-minute period of observation.

Phosphorylation of Signaling Proteins. Western blot analysis was used to measure changes in the phosphorylation of Akt (Ser473), p90RSK (Thr381), Stat 6 (Tyr641), and Erk1 and Erk2 (Thr183/Tyr185) in splenic B cells following stimulation with anti-IgM antibody, interleukin-4 (IL-4), or anti-CD40 antibody (Fig. 11). Stimulation with anti-IgM caused a rapid, increased phosphorylation of most proteins examined, except Stat 6. Maximal values were observed at 2.5 minutes, with Erk1 and Erk2 exhibiting the greatest increase. Anti-CD40 caused changes that mimicked those of anti-IgM, but the response was delayed until about 15 minutes after addition of ligand. By contrast, the response to IL-4 was characterized by a robust and sustained increase in the phosphorylation of Stat 6 (approximately 50-fold), while showing little or no effect on the other proteins. Phosphorylation of Akt followed a similar pattern of activation, but the increase was only 2.5-fold above that of untreated controls. These responses were reproduced in five independent experiments using different B-cell preparations.

Discussion

This report describes a standardized and reproducible procedure for the purification of splenic B cells from mice. The isolated cell population is composed of approximately 96% B cells defined by the expression of B220 and concomitant absence of CD43, Mac-1, and CD3. Of these, more than 80% can be further characterized as resting, mature B2 cells. Cells isolated in this manner maintain their viability over short-term culture in defined serum-free medium and demonstrate reproducible functional responses to ligands for cell-surface receptors.

The suitability of this population as a model for cellular signaling studies is supported by consistent and reproducible responses to several stimuli. A limitation of our approach, however, is that the cell population is not completely homogeneous. A similar limitation would apply to virtually any type of primary cell obtained from an animal. Fortunately, the heterogeneity of the B-cell population is not expected to affect the signaling responses measured by most assays. As we develop and refine our analyses in future experiments, it will be possible to address signaling differences between specific subpopulations (or even stochastic differences between individual cells of a population that appear genuinely homogeneous). For now, this population of cells is suitable for achieving our initial goal – that of identifying ligands that induce interesting signaling responses in B cells.

Methods and Protocols

Detailed methods and protocols used for these studies are available in PDF format and accessible via the online version of this report.

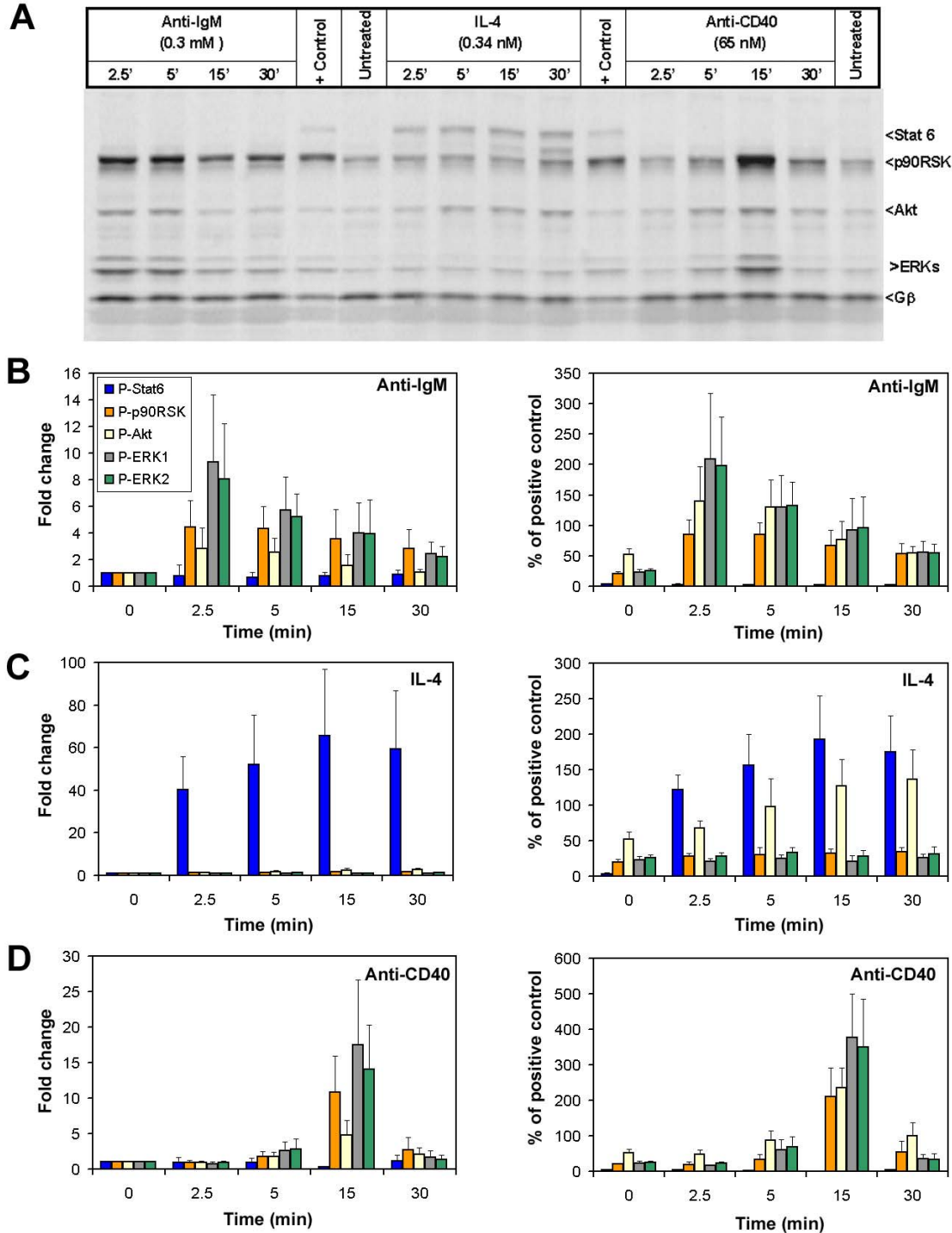


Fig. 11. Specific changes in protein phosphorylation induced by anti-IgM, interleukin 4 (IL-4), or anti-CD40. B cells were exposed to anti-IgM antibody (0.3 μM), IL-4 (0.34 nM), or anti-CD40 antibody (65 nM) for the indicated times. Cell extracts were analyzed by Western immunoblotting for changes in phosphorylation, first with a mixture of phosphospecific antibodies to Stat 6 (Tyr641), p90RSK (Thr381), Akt (Ser473), and Erk1 and Erk2 (Thr183/Tyr185). Following the initial processing, the blots were incubated with an antibody reactive to G protein β subunits 1 through 4 to correct for lane-to-lane variability in protein concentration. A representative blot is shown in panel (A). A positive control (+ Control) was prepared for use on all gels by exposure of a large number of cells to anti-IgM, IL-4, and protein phosphatase inhibitors. In panels (B-D) the quantification of Western blot signals is shown as the average ± SD for five independent experiments performed with as many separate preparations of B cells. The data were employed to calculate results as percent of positive control (bar graphs at right of each panel) or fold change (relative to the average for the samples of untreated cells; bar graphs at left of each panel).

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