The Journal of Immunology

Analysis of the Major Patterns of B Cell Gene Expression Changes in Response to Short-Term Stimulation with 33 Single Ligands

Xiaocui Zhu, Rebecca Hart, Mi Sook Chang, Jong-Woo Kim, Sun Young Lee, Yun Anna Cao, Dennis Mock, Eugene Ke, Brian Saunders, Angela Alexander, Joella Grossoehme, Keng-Mean Lin, Zhen Yan, Robert Hsueh, Jamie Lee, Richard H. Scheuermann, David A. Fruman, William Seaman, Shankar Subramaniam, Paul Sternweis, Melvin I. Simon and Sangdun Choi

J Immunol 2004; 173:7141-7149; ;
doi: 10.4049/jimmunol.173.12.7141
http://www.jimmunol.org/content/173/12/7141

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Analysis of the Major Patterns of B Cell Gene Expression Changes in Response to Short-Term Stimulation with 33 Single Ligands[1]

Xiaocui Zhu,* Rebecca Hart,* Mi Sook Chang,* Jong-Woo Kim,* Sun Young Lee,* Yun Anna Cao,* Dennis Mock,† Eugene Ke,† Brian Saunders,† Angela Alexander,§ Joella Grossoehme,§ Keng-Mean Lin,§ Zhen Yan,§ Robert Hsueh,§ Jamie Lee,¶ Richard H. Scheuermann,§¶ David A. Fruman,‖ William Seaman,# Shankar Subramaniam,†‡ Paul Sternweis,§ Melvin I. Simon,* and Sangdun Choi[2]*

We examined the major patterns of changes in gene expression in mouse splenic B cells in response to stimulation with 33 single ligands for 0.5, 1, 2, and 4 h. We found that ligands known to directly induce or costimulate proliferation, namely, anti-IgM (anti-Ig), anti-CD40 (CD40L), LPS, and, to a lesser extent, IL-4 and CpG-oligodeoxynucleotide (CpG), induced significant expression changes in a large number of genes. The remaining 28 single ligands produced changes in relatively few genes, even though they elicited measurable elevations in intracellular Ca^{2+} and cAMP concentration and/or protein phosphorylation, including cytokines, chemokines, and other ligands that interact with G protein-coupled receptors. A detailed comparison of gene expression responses to anti-Ig, CD40L, LPS, IL-4, and CpG indicates that while many genes had similar temporal patterns of change in expression in response to these ligands, subsets of genes showed unique expression patterns in response to IL-4, anti-Ig, and CD40L. *The Journal of Immunology,* **2004, 173: 7141–7149.**

The Alliance for Cellular Signaling recently completed a screen of the responses of mouse splenic B lymphocytes to the application of a panel of distinct single ligands in an attempt to determine: 1) which ligands will activate signaling pathways, and 2) how distinct are the ligands in their downstream signaling responses (1, 2). A total of 33 ligands was chosen for their known or suspected effects on purified mouse splenic B cells (see supplementary Table I[3] for the full list of the ligands). These ligands included those known to induce or costimulate proliferation, such as anti-Ig, CD40L, LPS, IL-4, and CpG (3–7). They also included several chemotactic ligands, including B cell-attracting chemokine 1 (BLC[4] or CXCL13), Ebl1-ligand chemokine (CCL19), secondary lymphoid-tissue chemokine (CCL21), and stromal cell-derived factor-1α (SDF1α or CXCL12), which mediate B cell migration at different stages of the B cell life cycle and are critical for proper lymphoid organ development, germinal center formation, and immune responses (8). In this study, we report the characteristics of B cell gene expression changes in response to these 33 ligands, as determined by custom Agilent (Agilent Technologies, Palo Alto, CA) two-color cDNA arrays. We identify the most potent ligands in inducing gene expression changes as those that promote proliferation (anti-Ig, CD40L, LPS, and, to a lesser extent, IL-4 and CpG). We also delineate the patterns of gene expression that are either shared by these ligands or are unique to the individual ligands.

Materials and Methods

Detailed information of procedures, ligands, and solutions used in the experiments can be found at the Alliance for Cellular Signaling protocols website: www.signaling-gateway.org/data/ProtocolLinks.html.

Mice, reagents, cell culture, and RNA preparation

Mice used in the experiments were 6- to 8-wk-old male C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME). Splenic B cells (>96% B220 positive) were purified from RBC-depleted splenocytes by using anti-CD43 and anti-Mac-1/CD11b mAbs coupled to magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), as described in Alliance for Cellular Signaling protocol PP00000016. Purified B cells were cultured with ligands or medium alone for 0.5, 1, 2, and 4 h, and their RNA was extracted following Alliance for Cellular Signaling protocol PP00000009. Triplicate experiments were performed for each ligand at each time point, and each RNA sample was processed and measured by a cDNA array. As

*Molecular Biology Laboratory, Alliance for Cellular Signaling, Division of Biology, California Institute of Technology, Pasadena, CA 91125; †Bioinformatics and Data Coordination Laboratory, Alliance for Cellular Signaling, San Diego Supercomputer Center, and ‡Department of Bioengineering, University of California, San Diego, CA 92122; §Cell Preparation and Analysis Laboratory, Alliance for Cellular Signaling, Department of Pharmacology, and ¶Department of Pathology, University of Texas Southwestern Medical Center, Dallas, TX 75390; ‖Department of Molecular Biology and Biochemistry, University of California, Irvine, CA 92697; and #Macrophage Biology Laboratory, Alliance for Cellular Signaling, Department of Medicine, University of California, San Francisco, CA 94143

Received for publication May 13, 2004. Accepted for publication September 21, 2004.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by contributions from public and private sources, including the National Institute of General Medical Sciences Glue Grant Initiative (U54 GM062114). A complete listing of the Alliance for Cellular Signaling sponsors can be found at www.signaling-gateway.org/aboutus/sponsors.html.

[2] Address correspondence and reprint requests to Dr. Sangdun Choi, Division of Biology, 147-75, California Institute of Technology, 1200 East California Boulevard, Pasadena, CA 91125. E-mail address: schoi@caltech.edu

[3] The on-line version of this article contains supplemental material.

[4] Abbreviations used in this paper: BLC, B cell-attracting chemokine 1; AKT, protein kinase B; Ccnd2, cyclin D2; Hk2, hexokinase 2; Ldh, lactate dehydrogenase; Ltb, lymphotoxin β; MARCKS, myristoylated alanine-rich protein kinase C substrate; PAF, platelet-activating factor; Plk, polo-like kinase; SAM, Significance Analysis of Microarrays; SDF, stromal cell-derived factor; Xbp, X box-binding protein.

0022-1767/04/$02.00

a reference for the cDNA array hybridization, RNA from total RBC-depleted splenocytes was extracted in the same manner as RNA from purified splenic B cells.

Agilent cDNA array fabrication and annotation

A total of 15,494 cDNA probes was printed on 15,832 spots on the custom-made arrays. Ninety-six percent of the probes came from the Riken Fantom collection (http://fantom.gsc.riken.jp; Yokohama, Kanagawa, Japan), with the rest from collections of the National Institute on Aging (Bethesda, MD), Research Genetics (Huntsville, AL), and Incyte Genomics (St. Louis, MO). The probes were PCR amplified by the Simon Lab at the California Institute of Technology (Pasadena, CA) and were inkjet printed onto glass slides by Agilent. The entire collection of the cDNA probes represents 10,615 unique genes as of the annotation results generated on December 30, 2003. See Table II in the supplemental material for the annotation table and a description of the annotation process.

Agilent cDNA array analysis

Each array was hybridized with Cy5-labeled cDNA prepared from the RNA of splenic B cells and Cy3-labeled cDNA prepared from RNA of total splenocytes that was used as an internal reference (Alliance for Cellular Signaling protocol PP00000019). The arrays were scanned using Agilent Scanner G2505A with the scan resolution set to 10 μm and the laser intensity adjusted so that both the maximum red and green (Cy5 and Cy3) fluorescence intensity were ~20,000 pixels. The image files were extracted with background-subtraction (the Local background subtraction method) and dye-normalization (the Rank consistent filter and the LOWESS algorithm) using the Agilent G2566AA Feature Extraction Software Version A.6.1.1. The entire raw data sets collected from over 400 arrays are available through the B cell ligand screen link at the Alliance for Cellular Signaling Data Center website: www.signaling-gateway.org/data/.

Data selection

For each array, we removed the control features. For features that were saturated (with Agilent glsSaturated and rlsSaturated flags), nonuniform (with Agilent gIsFeatNonUnifOL and rIsFeatNonUnifOL flags), or below background (with Agilent gIsWellAboveBG and rIsWellAboveBG flags), their Cy5 and Cy3 fluorescence intensity and $\log_2$ (Cy5/Cy3) value were set to blank. Features were further filtered by a statistical method, Significance Analysis of Microarrays (SAM) (9). Features identified by SAM as differentially expressed between treated and time-matched controls were included for downstream analysis. Our inputs for SAM analysis were measurements of the expression level of array features in B cells, represented as the Cy5 fluorescence intensity, which has been background subtracted and normalized for dye bias and interarray variance (see supplementary material for details of data preparation for SAM analysis). Features with fewer than two nonblank replicate measurements in treated or time-matched control B cells were not used in SAM analysis. One hundred random permutations were done for each comparison. A total of 3396 features was identified by SAM as differentially expressed in at least 1 of the 132 conditions with varied levels of false discovery rate. Of these, 459 features appeared to show changes in expression that correlated with the dates of RNA sample preparation and were excluded from downstream analysis.

Data clustering and identification of gene expression signatures

The input for clustering analysis was $\log_2$ (treated/0 h) value, the fold change of gene expression level in cultured B cells relative to that in 0-h B cells in $\log_2$ scale. The $\log_2$ (treated/0 h) value was calculated by subtracting $\log_2$ (0 h/spleen) from $\log_2$ (treated/spleen), in which the $\log_2$ (treated/spleen) is the average of at least two of three replicate $\log_2$ (Cy5/Cy3) measurements of cultured B cells and $\log_2$ (0 h/spleen) the average of at least 6 of the 26 replicate $\log_2$ (Cy5/Cy3) measurements of 0-h B cells. For features that did not have at least 2 replicate measurements in cultured B cells or at least 6 replicate measurements in 0-h B cells, their $\log_2$ (treated/0 h) value was set to blank (see supplementary Table III for $\log_2$ (treated/0 h) values of all 2937 differentially expressed features, and supplementary Table IV for $\log_2$ (treated/0 h) values of all array features). For each clustering analysis, only features that 1) were differentially expressed in at least one of the conditions, 2) had a nonblank $\log_2$ (treated/0 h) value in 80% of the conditions, and 3) whose maximum and minimum expression changes across the conditions had a difference greater than 1 were included.

To visualize the overall characteristics of gene expression profiles in response to the 33 single ligands or all the nonmitogenic ligands, hierarchical clustering was performed with the Cluster and TreeView program (10). Clustering was done one way across the features following gene-wise median centering, with experimental conditions aligned in ligand (alphabetical) and time-course orders. Euclidean correlation coefficient and complete linkage were used as similarity metrics.

To identify gene expression signatures in response to anti-Ig, CD40L, CpG, IL-4, or LPS, we first conducted K-means clustering of gene expression change over the 0.5-, 1-, 2-, and 4-h time course for each ligand to group features according to their temporal expression pattern. Then by comparing gene expression patterns across different ligand responses, we identified groups of features that showed unique expression changes in response to individual single ligands, and those that showed similar expression change patterns in response to all ligands. The groupings were further refined by removing features that had different expression patterns from the majority in the same group as revealed by hierarchical clustering using similar settings described above. K-means clustering and hierarchical clustering used in this study were implemented in the Multiple Experiment Viewer (www.tigr.org/software/tm4/mev.html).

Robust expression changes induced by BLC, IFN-β, IL-10, platelet-activating factor (PAF), and SDF1α and the signature expression change patterns induced by anti-Ig, CD40L, CpG, IL-4, and LPS were vizualized by hierarchical clustering implemented in the Multiple Experiment Viewer (www.tigr.org/software/tm4/mev.html). Clustering was done one way across the features following gene-wise median centering. Euclidean correlation coefficient and complete linkage were used as similarity metrics.

Quantitative real-time PCR

A total of 1 μg of total RNA from pooled duplicate or triplicate RNA samples used for microarray experiments was treated with DNase I (Invitrogen Life Technologies, Carlsbad, CA) to remove contaminating genomic DNA. First-strand cDNA was synthesized with SuperScript II (Invitrogen Life Technologies) and random hexamer priming (Invitrogen Life Technologies). Quantitative real-time PCR was performed by using i-Cycler (Bio-Rad, Hercules, CA) and SYBR green detection. See supplementary material for details of quantitative real-time PCR primers and the amplicon length of selected genes. Reactions were performed in 25 μl in triplicate wells in 96-well plates with the following ingredients: 5′ and 3′ primers (200 nM each), iQ SYBR Green Supermix (Bio-Rad), and cDNA corresponding to 40 ng of total RNA. Mouse β-actin was amplified in separate reactions in the same plate to be used as an internal control for variances in the amount of cDNA in PCR. See supplementary Table VIII for primer sequences and amplicon lengths. PCR cycle setup was as follows: 95°C for 1 min, 95°C for 3 min, 40× (95°C for 30 s, 60°C for 10 s, 72°C for 10 s). The Pfaffl's equation (11) was used to calculate the expression ratio (R) of ligand-treated vs control B cells. Briefly: $R = ((E_{target})^{\Delta CP}$ target (control − treated))/$((E_{\beta\text{-actin}})^{\Delta CP}$ β-actin (control − treated)), where $E = 10^{(-1/slope)}$, with the slope being the slope of the standard curve of the target genes or β-actin; ΔCP target (control − treated) is the average cross point cycle number of the control minus that of the treated sample for the target gene; ΔCP β-actin (control − treated) is the average cross point cycle number of control minus the treated sample for β-actin.

Results

Experimental strategy

To examine gene expression changes induced by the 33 single ligands, we cultured purified mouse splenic B cells with ligands in serum-free medium for 0.5, 1, 2, and 4 h. cDNA synthesized from the RNA of B cells was labeled with Cy5 and hybridized onto custom-made two-color Agilent cDNA arrays with a Cy3-labeled cDNA prepared from the RNA of total splenocytes. The gene expression change relative to 0 h in response to ligand or medium alone was then calculated by subtracting $\log_2$ (0 h/spleen) from $\log_2$ (treated/spleen). We had three considerations when choosing this experimental design. First, we focused on early gene expression changes with the rationale that these changes are likely to be regulated by signaling pathways directly activated downstream of receptor engagement and, therefore, best reflect the characteristics of ligand-specific signal transduction. Second, we used serum-free medium to avoid signaling events that might be triggered by serum growth factors. Finally, we used the RNA of total splenocytes as the universal reference in the two-color

cDNA array hybridization to control for the variance introduced during different times of array processing and to allow easy cross-condition comparisons (12).

Overview of characteristics of B cell gene expression profiles

The majority of the early gene expression changes were found in the responses to anti-Ig, CD40L, CpG, IL-4, and LPS, ligands that either directly induce or costimulate proliferation of resting B cells. Of the 2937 features identified by SAM analysis to be differentially expressed in response to the 33 single ligands during the 4-h period, 2625 features changed expression in response to anti-Ig, CD40L, CpG, IL-4, or LPS, while 797 features showed significant changes in response to the other 28 ligands. The dendrogram of the hierarchically clustered gene expression changes induced by all 33 single ligands revealed that the dominant gene expression pattern (Fig. 1*A*) shown by the intense red or green vertical strips was similar in the anti-Ig, CD40L, and LPS responses, representing coordinated up- or down-regulations of multiple genes. A similar pattern was detected in the CpG and IL-4 responses; however, the changes were of a smaller magnitude. No prominent expression pattern was observed in responses to the remaining 28 ligands (Fig. 1*A*). Because expression changes induced by the 28 ligands were of smaller magnitude than those induced by anti-Ig, CD40L, CpG, IL-4, and LPS, their patterns may be masked when all the expression changes are visualized together. To explore this possibility, we performed hierarchical clustering using only gene expression changes induced by the 28 ligands to look for significant patterns. We found that there were small clusters of genes that showed strong changes in response to BLC, SDF1α, IFN-β, IL-10, and PAF (Fig. 1*B*), while the rest of the genes were expressed in treated B cells at similar levels as they were in time-matched control B cells. See Fig. 2 for the expression changes of the genes in these small clusters.

Of the responses to anti-Ig, CD40L, LPS, CpG, or IL-4, the number of differentially expressed features identified by SAM analysis increased with time, with a sharp jump at 4 h (Fig. 3). A total of 2547 features changed expression at 4 h in contrast to a

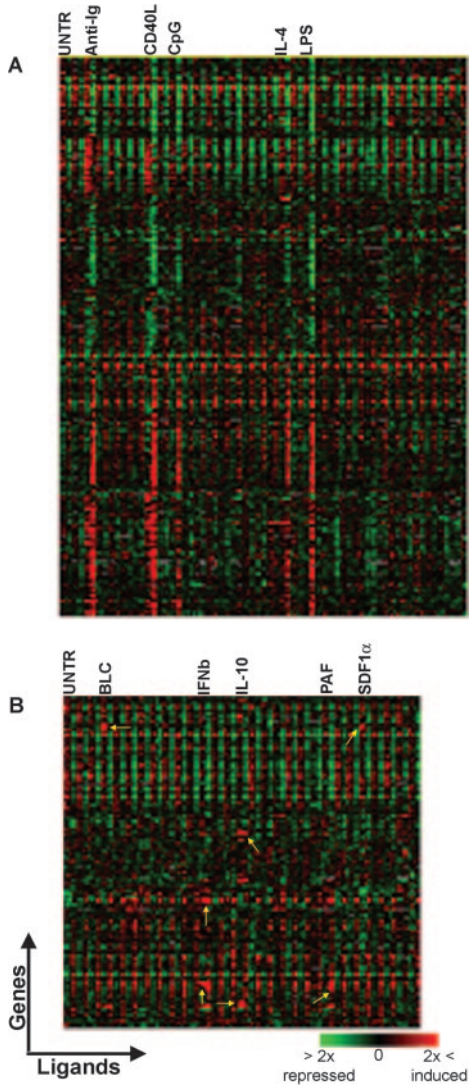

FIGURE 1. Dendrograms of hierarchically clustered expression changes induced by single ligands. Experimental conditions were aligned along the horizontal axis, in which the medium alone (UNTR) time course was followed by the time course of single ligands arranged according to the ligand's alphabetical order. Each time course consists of four time points: 0.5, 1, 2, and 4 h. Hierarchically clustered expression changes, calculated as the ratio of expression level in cultured B cells at 0.5, 1, 2, and 4 h vs that in 0-h B cells in the $\log_2$ scale, were displayed along the vertical axis. For details of data filtering, processing, and clustering, see *Material and Methods*. *A*, Gene expression changes induced by all 33 single ligands. UNTR and ligands promoting proliferation were labeled. *B*, Gene expression changes induced by 28 nonmitogenic ligands. The 28 ligands include all single ligands, except anti-Ig, CD40L, CpG, IL-4, and LPS. Yellow arrows point to examples of small clusters of strong expression changes. UNTR and ligands inducing small clusters of changes were labeled.

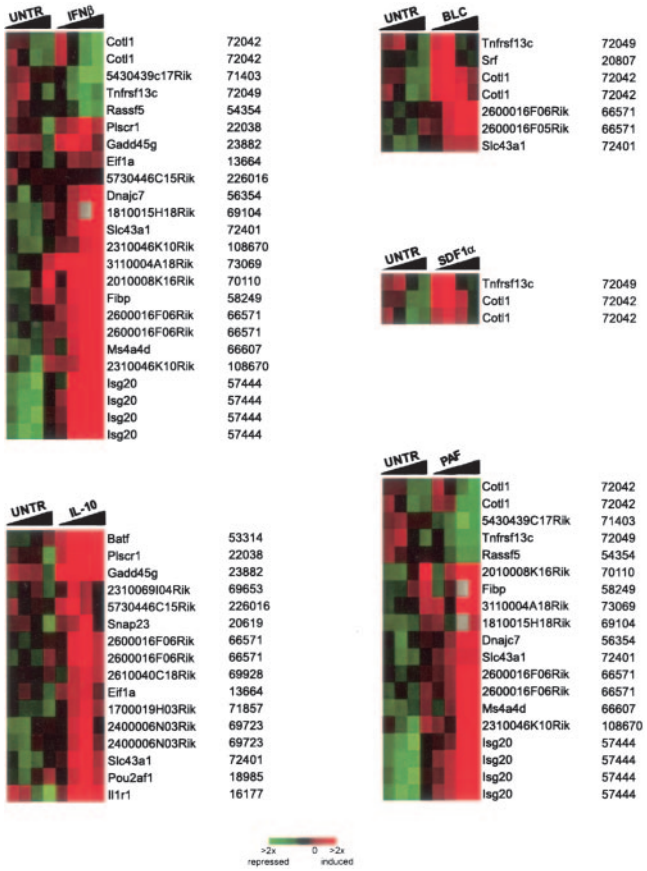

FIGURE 2. Features showing robust expression changes in response to BLC, IFN-β, IL-10, PAF, and SDF1α. The symbol and LocusLink of the genes are listed on the *right side* of the dendrogram. Each ▲ under the ligand labels represents a time course (0.5, 1, 2, and 4 h). Gene expression changes in B cells cultured in medium alone (UNTR) were included as the controls.

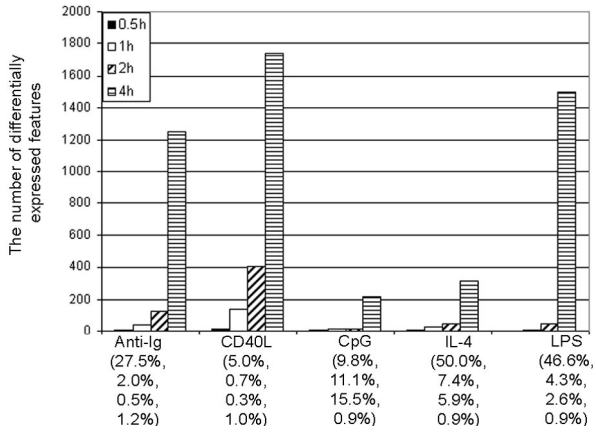

FIGURE 3. The number of differentially expressed features identified by SAM in response to anti-Ig, CD40L, CpG, IL-4, and LPS. The *y*-axis scales the number of differentially expressed features, and *x*-axis is for ligand condition. The false discovery rate of the differentially expressed feature list at each time point (0.5, 1, 2, and 4 h) is listed in the parenthesis.

total of 663 features at 0.5, 1, and 2 h. A previous study of anti-Ig response in mouse splenic B cells also reported a sharp increase at 4 h in the number of affected genes (13). The kinetics of expression change varied in response to different ligands. At 0.5, 1, and 2 h, anti-Ig and CD40L induced greater numbers of changes than LPS, CpG, and IL-4 (Fig. 3), and by 4 h, a large number of genes changed expression in response to LPS, anti-Ig, and CD40L, while considerably fewer genes were affected by CpG and IL-4. Thus, anti-Ig and CD40L induced the most immediate early gene expression changes, but by 4 h, anti-Ig, CD40L, and LPS all had extensive effects on gene expression, with lesser effects by CpG and IL-4.

The finding that the five proliferative ligands induced the strongest gene expression changes among the 33 ligands led us to focus on the proliferative ligands to examine more closely the similarities and differences in their downstream gene expression responses. We sought to identify target genes that were uniquely regulated by individual ligands or were coregulated by a group of ligands. Four major expression patterns were identified in anti-Ig, CD40L, CpG, IL-4, and LPS responses to date. We report in this study the genes in each distinct pattern and, when information is available, speculate on the pathways of signal transduction and on the biological relevance of the expression changes observed.

Expression change patterns shared by anti-Ig, CD40L, IL-4, CpG, and LPS responses

A large number of features showed a similar temporal pattern of expression change in response to anti-Ig, CD40L, CpG, IL-4, or LPS, while the magnitude of change varied from ligand to ligand for some features. A total of 176 features (149 unique genes) decreased and 294 features (228 unique genes) increased their expression at least 30% in response to treatment with each of the five ligands, relative to culture in medium (Fig. 4). See supplementary Table V for expression fold changes of up-regulated and down-regulated features. Although strong expression changes were observed for some of the features at 2 h, almost all of the features exhibited significant up- or down-regulation at 4 h. Several genes regulating cell cycle entry and progression showed expected expression changes. For example, cyclin-dependent kinase 4, a gene promoting cell cycle progression, was up-regulated (Fig. 4*B*); Forkhead box O1 and cyclin G_2, genes inhibiting cell cycle entry, were down-regulated by the ligands. So was a transcription repressor, basic Kruppel-like factor 3 (Fig. 4*A*) (14–19). Several genes involved in signal transduction decreased expression with time in response to the ligands. These include MAPK kinase 6, a kinase that directly activates p38 MAPK (20); spleen tyrosine kinase, a kinase that contributes to the activation of Bruton's tyrosine kinase and phospholipase C-γ1 downstream of BCR (21); and the α isoform of diacylglycerol kinase, a kinase suggested to attenuate receptor signaling in T cells by converting a secondary messenger, diacylglycerol, to phosphatidic acid (22) (Fig. 4*A*). These changes in gene expression may result in subsequent changes in cell signaling responses, although the precise role of such gene expression changes in regulating B cell activity is not clear.

We also found that several genes encoding enzymes of the glycolysis pathway, including hexokinase 2 (Hk2), phosphoglycerate mutase 1, lactate dehydrogenase 2 (Ldh2), and lactate dehydrogenase 3 (Ldh3), were up-regulated. Glycolysis converts the 6-carbon glucose into 3-carbon pyruvate and NADH. Pyruvate is either oxidized further through the TCA cycle into CO_2 and H_2O in the mitochondria aerobically, or is reduced by NADH to lactate anaerobically in the cytosol. Hk2 and phosphoglycerate mutase 1 proteins catalyze two different steps of the pathway that leads to the generation of pyruvate, while Ldh2 and Ldh3 proteins reduce pyruvate to lactate. The up-regulation of these glycolysis genes suggests that glycolysis activity increases and shifts toward the anaerobic pathway in B cells stimulated with ligands promoting cell growth and proliferation. In fact, several reports showed that lymphocyte cell lines and primary cells up-regulated the activity of multiple glycolysis enzymes including Hk2, and increased glycolysis rate and lactate production, when stimulated with cytokines or other mitogenic ligands such as anti-CD3 plus anti-CD28 (23, 24). Although glycolysis is generally thought to be regulated by metabolic demands of the cells through feedback mechanisms, these recent reports suggest that glycolysis activity can be directly regulated by signal transduction pathways downstream of the receptors, such as the PI3K/protein kinase B (AKT) pathway (23, 24). The shift toward anaerobic glycolysis is not because the cells lack the ability to undergo TCA cycle for maximal energy output, but rather is due to the fact that the rate of pyruvate and NADH generation from the high glycolysis activity and the ample supply of glucose in the culture medium exceeds the rate of their consumption in the mitochondria. As a result, pyruvate is reduced by NADH to lactate and secreted by the cells (23, 24). In vivo when nutrients such as glucose are limited, the up-regulation of glycolysis by mitogenic signals can help stimulated B cells to mount rapid cell growth and proliferation and allow them an advantage in using nutrients over unstimulated cells.

Finally, consistent with a previous study on anti-Ig gene expression response in B cells (25), we found that a large number of genes (64 features representing 46 unique genes) involved in mRNA processing and translation were almost exclusively up-regulated. These expression changes are likely to lead to the up-regulation of protein synthesis, which is required for cell size increase, DNA replication, and cell division. See supplementary Table V for expression fold changes of genes involved in RNA processing and translation.

Gene expression change patterns distinguishing IL-4

Genes in this group exhibited two expression change patterns: 1) not changed by anti-Ig, CD40L, CpG, and LPS, while strongly up-regulated by IL-4 (Fig. 5*A*), and 2) down-regulated by anti-Ig, CD40L, CpG, and LPS, while up-regulated or not changed by IL-4 (Fig. 5*B*). See supplementary Table VI for expression fold changes of features of known genes in this group. Three of the genes uniquely up-regulated by IL-4, Caspase-6, X box-binding protein

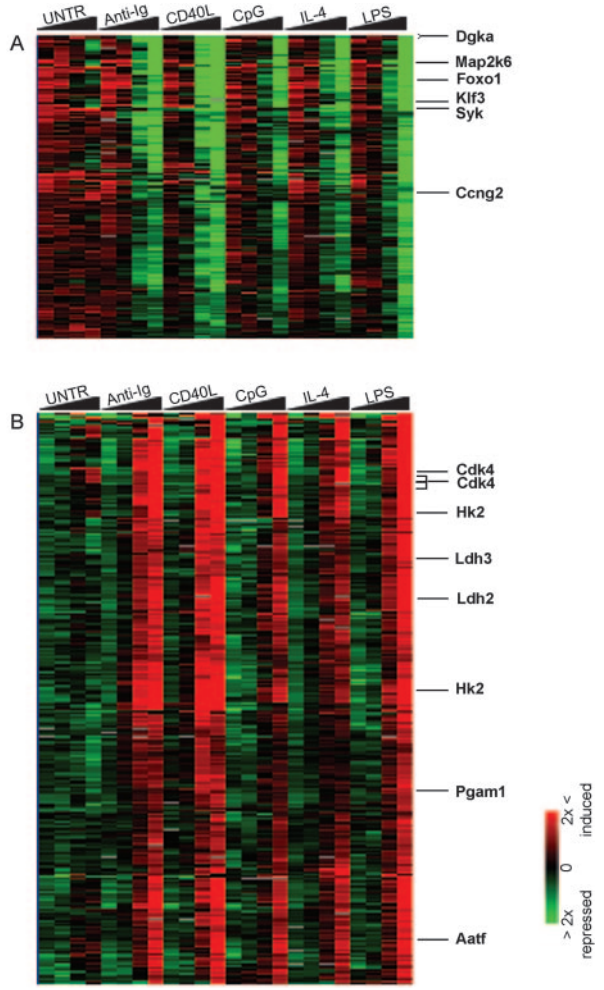

FIGURE 4. Features showing similar temporal expression change patterns in response to anti-Ig, CD40L, CpG, IL-4, and LPS. *A*, Features that decreased expression with time. *B*, Features that increased expression with time. Each ▲ under the ligand labels represents a time course (0.5, 1, 2, and 4 h). Gene expression changes in B cells cultured in medium (UNTR) alone were included as the controls. The symbol of genes discussed in the results is labeled on the *right side* of the dendrogram.

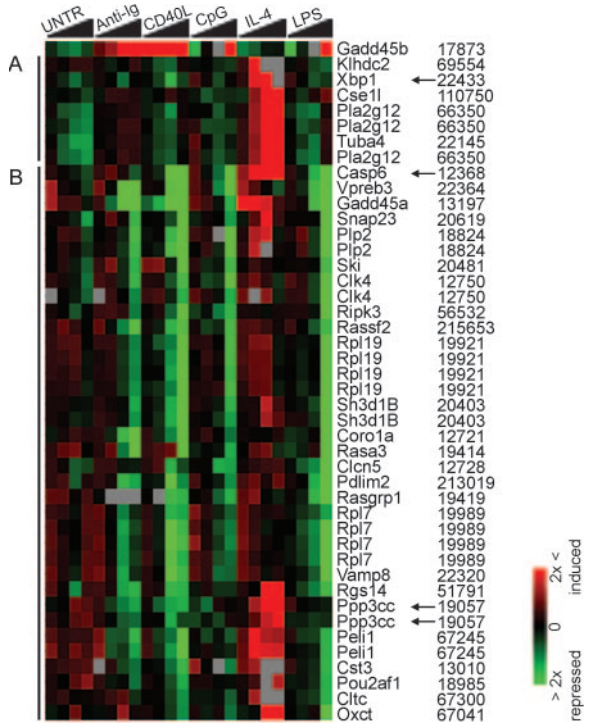

FIGURE 5. Features exhibiting IL-4-specific expression patterns. *A*, Features strongly up-regulated by IL-4, while not changed by anti-Ig, CD40L, CpG, and LPS. *B*, Features strongly up-regulated or not changed by IL-4, while down-regulated by anti-Ig, CD40L, CpG, and LPS. Only features representing known genes are shown. The symbol and LocusLink of the genes are listed on the *right side* of the dendrogram. Genes discussed in the results are pointed to by arrows. Each ▲ under the ligand labels represents a time course (0.5, 1, 2, and 4 h). Gene expression changes in B cells cultured in medium alone (UNTR) were included as the controls.

1 (Xbp-1), and the γ isoform of calcineurin catalytic subunit have been suggested to be STAT6 targets in B cells and/or T cells in published reports (26–28). Additional experiments are needed to determine whether other genes up-regulated by IL-4 are also transcriptional targets of STAT6. For genes whose expression was not changed by IL-4 while down-regulated by other ligands, the simplest explanation could be that they are target genes of transcription repressors activated by anti-Ig, CD40L, CpG, LPS, but not by IL-4. It is also possible that these are target genes of transcription factors inactivated by anti-Ig and others, but not by IL-4.

We are only beginning to understand the biological relevance of the IL-4-specific gene expression pattern. Xbp-1, which was strongly up-regulated within 1 h of IL-4 stimulation, is a crucial transcriptional factor for plasma cell differentiation (29). Intriguingly, Xbp-1 activation requires an mRNA splicing event triggered by the unfolded protein response, which leads to a reading frame shift and subsequent translation of an additional *trans* activation domain (30–32). It was shown that while IL-4 was the only cytokine that rapidly induces Xbp-1 in mouse B cells, long-term treatment with anti-CD40 or LPS, up to 2 days, strongly induced Xbp-1 mRNA processing as well as transcription (28, 29, 33). Increased load of Ig in endoplasmic reticulum in response to anti-CD40 and LPS may initiate the unfolded protein response and subsequent Xbp-1 mRNA splicing. Indeed, Xbp-1 mRNA splicing depends on IgM protein synthesis during B cell differentiation, and the spliced form is in turn required for Ig production and secretion (28, 34). Xbp-1 transcripts induced early by IL-4 may help to ensure efficient processing of subsequently produced Ig, and may have other as yet unknown biological significances (28). No change in Xbp-1 expression in response to IL-4 was detected in mouse T cells (27), suggesting that cell type-specific proteins and possibly chromosome accessibility are involved in regulating Xbp-1 expression. With regard to cell proliferation, we did not find the induction of proliferation inhibitors or the lack of expression of genes required for proliferation that can account for the lack of direct proliferative effect of IL-4 compared with anti-Ig, CD40L, CpG, and LPS. It is possible that quantitative characteristics in gene expression in response to IL-4 could contribute to the lack of proliferative effect of IL-4, notably, the generally smaller magnitude of expression changes (Fig. 3). Our results also suggest that the ability of IL-4 to promote proliferation as a cofactor is not solely attributed to the up-regulation of antiapoptotic genes, such as Bcl-2, by IL-4 stimulation (35), but it may be due to the synergistic induction of many genes involved in cell proliferation (Fig. 4).

Gene expression change patterns distinguishing anti-Ig

Genes in this group demonstrate mainly two temporal expression patterns: 1) strongly up-regulated by CD40L, IL-4, CpG, and LPS, while not changed or only slightly up-regulated by anti-Ig (Fig.

6*A*), and 2) down-regulated by other ligands, mainly at 2 and 4 h, while up-regulated by anti-Ig (Fig. 6*B*). See supplementary Table VII for expression fold changes of features of known genes in this group. Worth noting in Fig. 6*A* is the significantly weaker up-regulation of two genes required for cell cycle entry and progression, namely cyclin D2 (Ccnd2) and polo-like kinase (Plk), in response to anti-Ig compared with that in response to CD40L, IL-4, CpG, and LPS (36–38). The comparatively modest increase in Ccnd2 and Plk expression could reflect heterogeneity in the splenic B cell population; although most splenic B cells are mature follicular cells, they also include transitional immature cells and mature marginal zone cells (39, 40). It is known that immature B cells undergo apoptosis and down-regulate Ccnd2 in response to anti-Ig (39, 41, 42), but do proliferate to the other mitogen, LPS (39). It is possible that different gene response patterns to anti-Ig in the B cell subsets could dilute or cancel each other out at the population level, contributing to less up-regulation of Ccnd2 and Plk. This explanation also fits with the observation that mouse splenic B cells showed more death in response to anti-Ig than to CD40L or LPS (43–45). Another gene that was strongly induced by CD40L, CpG, IL-4, and LPS, but not by anti-Ig, is myristoylated alanine-rich protein kinase C substrate (MARCKS). MARCKS encodes a membrane protein that can bind to members of protein kinase C family proteins, actin, and the calmodulin-Ca^{2+} complex (46, 47). It was proposed that the MARCKS protein can bind and sequester a significant fraction of phosphoinositol-4,5-biphosphate in lateral membrane domains and can release the lipid when phosphorylated by protein kinase C family members or when it binds to calmodulin-Ca^{2+} complex (48–50). Thus, the increased expression of MARCKS may affect signaling by inositol phosphates.

Several genes involved in endocytosis and intracellular traffic were up-regulated by anti-Ig and down-regulated by other ligands (Fig. 6*B*). Atp6ap2, Atp6v0e, and Atp6v1c1 encode subunits of H^+-ATPases whose function is to transport H^+ from cytoplasm into intracellular compartments, resulting in luminal acidification, which is important in regulating various steps of endocytosis and intracellular traffic (51). Other genes up-regulated by anti-Ig that may be involved in endocytosis and intracellular trafficking include sorting nexin 5, a member of the nexin family proteins postulated to be involved in intracellular protein targeting (52), and chloride channel 7, a member of the voltage-gated chloride channel family proteins that have been suggested to contribute to luminal acidification by charge neutralization (53). It has been shown that BCR signaling facilitates multiple steps of the Ag presentation pathway, in which BCR-Ag complexes are internalized and targeted to the endosomal compartment, followed by Ag processing and loading onto MHC class II complex, and finally presentation on the cell surface (54–58). Increasing the expression of genes involved in endocytosis and intracellular traffic may be one of the mechanisms by which BCR signaling accelerates Ag presentation, which is important for B cell expansion and selection in vivo.

Nfatc1, encoding the C1 subunit of the NF-AT transcription factor, was up-regulated at 2 and 4 h in response to anti-Ig while remaining unchanged in response to other proliferative ligands

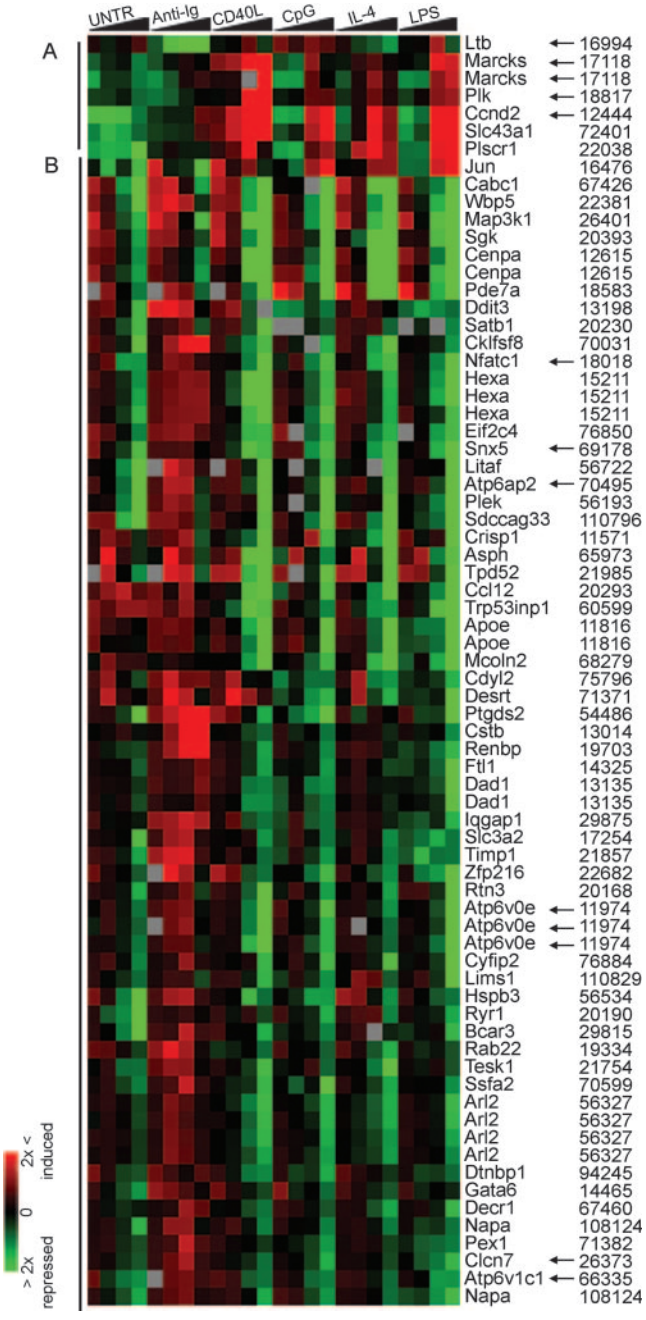

FIGURE 6. Features showing anti-Ig-specific expression patterns. *A*, Features exhibited weaker up-regulation in response to anti-Ig than to CD40L, CpG, IL-4, and LPS. *B*, Features up-regulated by anti-Ig and down-regulated or not changed by CD40L, CpG, IL-4, and LPS. The description of the figure is the same as in Fig. 5.

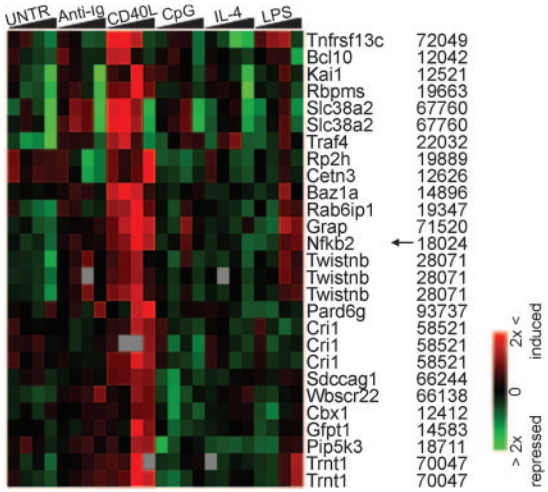

FIGURE 7. Features up-regulated by CD40L and not changed by anti-Ig, CpG, IL-4, and LPS. The description of the figure is the same as in Fig. 5.

(Fig. 6*B*). This observation, together with the fact that anti-Ig activates NF-AT, raises the possibility that Nfatc1 expression may be regulated by a positive feedback loop, in which activated NF-AT transcription factor induces Nfatc1 expression (59). This mechanism may sustain NF-AT activation by anti-Ig. Lymphotoxin β (Ltb) was strongly down-regulated by anti-Ig (Fig. 6), an unexpected result given that Ltb produced by B cells is required for normal T-dependent humoral responses (60). A smaller magnitude of down-regulation was induced by IL-4. Whether Ltb expression is up-regulated in later time points beyond 4 h is unknown. CD40L was previously shown to induce strong and sustained up-regulation of Ltb in B cells following 24 h of stimulation (61). It is possible that costimulation with CD40L is required to up-regulate Ltb in B cells during T-dependent immune response.

Gene expression changes distinguishing CD40L

Genes in this group in general exhibited greater up-regulation in response to CD40L than in response to other ligands (Fig. 7). See supplementary Table VIII for expression fold changes of features of known genes in this group. Of particular interest is Nfκb2, which showed strong and consistent up-regulation in response to CD40L during the 4-h period. NF-κB2 is required for peripheral B cell maintenance and for normal development of secondary lymphoid tissue architecture and humoral responses (62, 63). Several reports demonstrated that NF-κB2, together with its binding partner, RELB, is activated via an alternative pathway, whereby extracellular stimuli such as the B cell-activating factor of the TNF family, CD40L, and LTβ induce the processing of the p100 precursor form of NF-κB2 into p52, which subsequently translocates into the nucleus with RELB to activate transcription (64–68). It is conceivable that the increased expression of Nfκb2 in response to CD40L may contribute to sustained activation of NF-κB2-RELB pathway; however, the precise function of the pathway in CD40L-mediated B cell activation remains to be defined.

Results of quantitative real-time PCR validation of selected microarray results

To validate the microarray data, we measured expression changes of seven of the genes we discussed in our paper in response to 4-h treatment with anti-Ig, CD40L, and IL-4 using quantitative real-time PCR. As shown in Fig. 8, gene expression changes measured by quantitative real-time PCR are in general comparable to those determined by microarray. PCR measured Ltb expression changes induced by anti-Ig, CD40L, and IL-4; Ntac1 expression change induced by CD40L and IL-4; and Xbp-1 expression change induced by anti-Ig were greater than those measured by microarray. In three cases, PCR measured expression change of Xbp-1 induced by CD40L, Atp6ap2 expression change induced by IL-4, and Nfκb2 expression changes induced by anti-Ig are in a different direction than those determined by microarray. In these cases, the expression changes (in $\log_2$ scale) measured using both approaches are all smaller than 0.5. It is generally thought that measurement of smaller expression changes by microarray tends to be more variable. Despite the above-mentioned differences, ligand-specific expression change patterns determined by quantitative real-time PCR are largely consistent with those revealed by microarray data. Specifically, spleen tyrosine kinase showed up-regulation in response to all three ligands; γ isoform of calcinerin catalytic subunit and Xbp-1 showed the strongest up-regulation in response to IL-4, while showing little change or down-regulation in response to anti-Ig and CD40L; Atp6ap2 and Nfatc1 were uniquely up-regulated by anti-Ig, while showing little change or down-regulation in response to CD40L and IL-4; Ltb showed the strongest down-regulation in response to Anti-Ig; and finally, Nfκb2 was strongly up-regulated by CD40L.

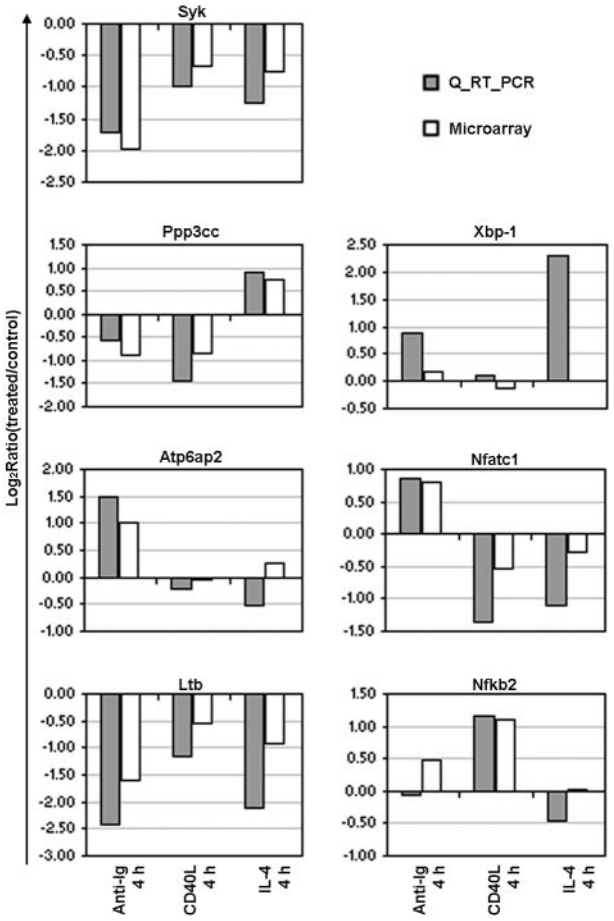

FIGURE 8. Validation of the expression of selected genes in different expression patterns using quantitative real-time PCR. Duplicate or triplicate total RNA samples used for microarray experiments of 4-h untreated, 4-h anti-Ig, 4-h CD40L, and 4-h IL-4-treated B cells were pooled, and 1-μg aliquots were reverse transcribed and used as templates to amplify selected genes by quantitative real-time PCR with SYBR green detection. The ratio of the expression level of each gene in treated vs untreated control B cells was determined with the Pfaffl method (see *Materials and Methods*), and its value in the $\log_2$ scale was plotted in ▨. Microarray-measured gene expression changes, $\log_2$(4-h treated/4-h untreated), were calculated by subtracting the average of triplicate $\log_2$(4-h untreated/0 h) from the average of triplicate $\log_2$(4-h treated/0 h) and were plotted in □.

Discussion

In the present study, we described the first study of genome-wide transcriptional responses in B cells to short-term stimulation with a comprehensive set of 33 single ligands, almost all of which induce measurable early upstream biochemical signals. Our first finding is that 5 of the 33 ligands that directly induce or costimulate proliferation generated most of the significant gene expression changes, while the remaining 28 ligands, many of which produce short-term reversible responses, such as chemokines, tended not to induce sustained robust gene expression changes. On one hand, it makes sense that proliferative ligands need to induce immediate early gene expression changes that can further drive cellular changes required for proliferation, such as the increase of cell size, DNA replication, and cell division. In contrast, chemotactic ligands, which generate only transient morphological changes that allow cells to migrate along a chemokine gradient, need to avoid

inducing expression changes that would lead to long-term irreversible cellular alterations. On the other hand, the finding is intriguing given the fact that chemotactic ligands, BLC, Ebl1-ligand chemokine, secondary lymphoid-tissue chemokine, and SDF1α, induced significant Ca^{2+} flux, cAMP generation, and phosphorylation of AKT, ERK1/2, and P90RSK, while mitogenic ligand CpG induced little or no Ca^{2+} flux, cAMP generation, and limited kinase phosphorylation (data available through the B cell ligand screen link at the Alliance for Cellular Signaling Data Center website www.signaling-gateway.org/data/). It appears then that the mere activation of an upstream kinase or other signaling events does not necessarily lead to the extensive expression of its downstream target genes. In addition, a strong signature of classical signaling pathways is not always required for robust gene expression changes. It is likely that gene expression is regulated by multiple upstream inputs, including, but not limited to, the ones measured in our study, in which the timing, duration, as well as intensity of each input could possibly have synergistic effects.

Our second finding is that a large number of genes showed similar temporal patterns of expression change in response to anti-Ig, CD40L, CpG, IL-4, and LPS, while a few genes exhibited patterns unique for anti-Ig, CD40L, or IL-4. It is intriguing that receptors with distinct signal transduction pathways induced largely overlapping early gene expression change profiles. Anti-Ig, CD40L, IL-4, and CpG/LPS receptors recruit unique kinases and/or adaptor proteins to the membrane immediately downstream of ligand engagement. Through intermediate steps that are not completely understood, unique as well as common signaling events are induced further downstream. Although Ca^{2+} release and NF-AT are induced only by anti-Ig and STAT6 mostly by IL-4 (5–8, 69), AKT, NF-κB, PI3K, ERK 1 and 2 (ERK1/2), JNK, and p38 MAPK can be activated by all or a subset of the ligands. That distinct signal transduction pathways can affect the expression of a common set of immediate early genes has been reported before. It was found that in NIH 3T3 cells, mutating the binding sites for activation of the phospholipase C-γ1, PI3K, Src homology region 2 domain-containing phosphatase 1, and Ras GTPase-activating protein of platelet-derived growth factor β receptor only led to quantitative, but not qualitative change in the expression of a common set of immediate early genes in response to platelet-derived growth factor stimulation (70). This observation led to the suggestion that distinct signal transduction pathways, rather than regulate the expression of specific subsets of immediate early genes, generate largely overlapping effects on the immediate early genes (70, 71). This can be achieved by a mechanism whereby distinct signal transduction pathways activate multiple transcription factors, each or subsets of which can exert qualitatively similar effects on the expression of early genes. Other mechanisms include cross talks between signal transduction pathways and the convergence of different signals to common regulatory mechanisms, such as the MAPK pathways, which generate the shared early transcriptional response. These possibilities are not mutually exclusive; each of them can contribute to the overlapping effects of different ligands on early gene expression. In any case, the specificity of the biological functions of different ligands can be mediated by either qualitative differences in early gene expression and/or the unique induction or repression of small subsets of genes. In future work, combining these comprehensive gene expression change measurements with the signaling studies conducted with these ligands will allow a greater understanding about how signaling networks impact gene expression. These inferences can be further strengthened by perturbations induced in specific signaling pathways and by computational prediction of transcription factor binding sites in the promoters of shared and distinct target genes.

Note added in proof. The microarray data used in this paper were deposited into GEO (www.ncbi.nlm.nih.gov/geo/, Gene Expression Omnibus) under the accession numbers of GSM5420, GSM5422-5428, GSM5437-5552, GSM5556, GSM5558, GSM5567-5851, GSM15996-16100, and GSM16108-16164.

Acknowledgments

We thank Dr Gilberto R. Sambrano and other members of Alliance for Cellular Signaling for their critical review and insightful input during the preparation of this manuscript.

References

1. Sambrano, G. R., G. Chandy, S. Choi, D. Decamp, R. Hsueh, K. M. Lin, D. Mock, N. O'Rourke, T. Roach, H. Shu, et al. 2002. Unravelling the signal-transduction network in B lymphocytes. *Nature 420:708*.
2. Gilman, A. G., M. I. Simon, H. R. Bourne, B. A. Harris, R. Long, E. M. Ross, J. T. Stull, R. Taussig, A. P. Arkin, M. H. Cobb, et al. 2002. Overview of the Alliance for Cellular Signaling. *Nature 420:703*.
3. Kearney, J. F., and A. R. Lawton. 1975. B lymphocyte differentiation induced by lipopolysaccharide. I. Generation of cells synthesizing four major immunoglobulin classes. *J. Immunol. 115:671*.
4. DeFranco, A. L. 1993. Structure and function of the B cell antigen receptor. *Annu. Rev. Cell Biol. 9:377*.
5. Foy, T. M., A. Aruffo, J. Bajorath, J. E. Buhlmann, and R. J. Noelle. 1996. Immune regulation by CD40 and its ligand GP39. *Annu. Rev. Immunol. 14:591*.
6. Nelms, K., A. D. Keegan, J. Zamorano, J. J. Ryan, and W. E. Paul. 1999. The IL-4 receptor: signaling mechanisms and biologic functions. *Annu. Rev. Immunol. 17:701*.
7. Krieg, A. M. 2002. CPG motifs in bacterial DNA and their immune effects. *Annu. Rev. Immunol. 20:709*.
8. Sallusto, F., C. R. Mackay, and A. Lanzavecchia. 2000. The role of chemokine receptors in primary, effector, and memory immune responses. *Annu. Rev. Immunol. 18:593*.
9. Tusher, V. G., R. Tibshirani, and G. Chu. 2001. Significance analysis of microarrays applied to the ionizing radiation response. *Proc. Natl. Acad. Sci. USA 98:5116*.
10. Eisen, M. B., P. T. Spellman, P. O. Brown, and D. Botstein. 1998. Cluster analysis and display of genome-wide expression patterns. *Proc. Natl. Acad. Sci. USA 95:14863*.
11. Pfaffl, M. W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res. 29:e45*.
12. Novoradovskaya, N., M. L. Whitfield, L. S. Basehore, A. Novoradovsky, R. Pesich, J. Usary, M. Karaca, W. K. Wong, O. Aprelikova, M. Fero, et al. 2004. Universal Reference RNA as a standard for microarray experiments. *BMC Genomics 5:20*.
13. Glynne, R., S. Akkaraju, J. I. Healy, J. Rayner, C. C. Goodnow, and D. H. Mack. 2000. How self-tolerance and the immunosuppressive drug FK506 prevent B-cell mitogenesis. *Nature 403:672*.
14. Turner, J., and M. Crossley. 1998. Cloning and characterization of mCtBP2, a co-repressor that associates with basic Kruppel-like factor and other mammalian transcriptional regulators. *EMBO J. 17:5129*.
15. Turner, J., H. Nicholas, D. Bishop, J. M. Matthews, and M. Crossley. 2003. The LIM protein FHL3 binds basic Kruppel-like factor/Kruppel-like factor 3 and its co-repressor C-terminal-binding protein 2. *J. Biol. Chem. 278:12786*.
16. Machida, S., E. E. Spangenburg, and F. W. Booth. 2003. Forkhead transcription factor FoxO1 transduces insulin-like growth factor's signal to p27Kip1 in primary skeletal muscle satellite cells. *J. Cell. Physiol. 196:523*.
17. Bennin, D. A., A. S. Don, T. Brake, J. L. McKenzie, H. Rosenbaum, L. Ortiz, A. A. DePaoli-Roach, and M. C. Horne. 2002. Cyclin G_2 associates with protein phosphatase 2A catalytic and regulatory B′ subunits in active complexes and induces nuclear aberrations and a G_1/S phase cell cycle arrest. *J. Biol. Chem. 277:27449*.
18. Horne, M. C., K. L. Donaldson, G. L. Goolsby, D. Tran, M. Mulheisen, J. W. Hell, and A. F. Wahl. 1997. Cyclin G_2 is up-regulated during growth inhibition and B cell antigen receptor-mediated cell cycle arrest. *J. Biol. Chem. 272:12650*.
19. Martinez-Gac, L., M. Marques, Z. Garcia, M. R. Campanero, and A. C. Carrera. 2004. Control of cyclin G_2 mRNA expression by forkhead transcription factors: novel mechanism for cell cycle control by phosphoinositide 3-kinase and forkhead. *Mol. Cell. Biol. 24:2181*.
20. Moriguchi, T., N. Kuroyanagi, K. Yamaguchi, Y. Gotoh, K. Irie, T. Kano, K. Shirakabe, Y. Muro, H. Shibuya, K. Matsumoto, et al. 1996. A novel kinase cascade mediated by mitogen-activated protein kinase kinase 6 and MKK3. *J. Biol. Chem. 271:13675*.
21. Kurosaki, T. 1999. Genetic analysis of B cell antigen receptor signaling. *Annu. Rev. Immunol. 17:555*.
22. Sanjuan, M. A., D. R. Jones, M. Izquierdo, and I. Merida. 2001. Role of diacylglycerol kinase α in the attenuation of receptor signaling. *J. Cell Biol. 153:207*.
23. Frauwirth, K. A., J. L. Riley, M. H. Harris, R. V. Parry, J. C. Rathmell, D. R. Plas, R. L. Elstrom, C. H. June, and C. B. Thompson. 2002. The CD28 signaling pathway regulates glucose metabolism. *Immunity 16:769*.
24. Bauer, D. E., M. H. Harris, D. R. Plas, J. J. Lum, P. S. Hammerman, J. C. Rathmell, J. L. Riley, and C. B. Thompson. 2004. Cytokine stimulation of

aerobic glycolysis in hematopoietic cells exceeds proliferative demand. *FASEB J. 18:1303*.
25. Fruman, D. A., G. Z. Ferl, S. S. An, A. C. Donahue, A. B. Satterthwaite, and O. N. Witte. 2002. Phosphoinositide 3-kinase and Bruton's tyrosine kinase regulate overlapping sets of genes in B lymphocytes. *Proc. Natl. Acad. Sci. USA 99:359*.
26. Schroder, A. J., P. Pavlidis, A. Arimura, D. Capece, and P. B. Rothman. 2002. Cutting edge: STAT6 serves as a positive and negative regulator of gene expression in IL-4-stimulated B lymphocytes. *J. Immunol. 168:996*.
27. Chen, Z., R. Lund, T. Aittokallio, M. Kosonen, O. Nevalainen, and R. Lahesmaa. 2003. Identification of novel IL-4/Stat6-regulated genes in T lymphocytes. *J. Immunol. 171:3627*.
28. Iwakoshi, N. N., A. H. Lee, P. Vallabhajosyula, K. L. Otipoby, K. Rajewsky, and L. H. Glimcher. 2003. Plasma cell differentiation and the unfolded protein response intersect at the transcription factor XBP-1. *Nat. Immunol. 4:321*.
29. Reimold, A. M., N. N. Iwakoshi, J. Manis, P. Vallabhajosyula, E. Szomolanyi-Tsuda, E. M. Gravallese, D. Friend, M. J. Grusby, F. Alt, and L. H. Glimcher. 2001. Plasma cell differentiation requires the transcription factor XBP-1. *Nature 412:300*.
30. Shen, X., R. E. Ellis, K. Lee, C. Y. Liu, K. Yang, A. Solomon, H. Yoshida, R. Morimoto, D. M. Kurnit, K. Mori, and R. J. Kaufman. 2001. Complementary signaling pathways regulate the unfolded protein response and are required for *C. elegans* development. *Cell 107:893*.
31. Yoshida, H., T. Matsui, A. Yamamoto, T. Okada, and K. Mori. 2001. XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. *Cell 107:881*.
32. Calfon, M., H. Zeng, F. Urano, J. H. Till, S. R. Hubbard, H. P. Harding, S. G. Clark, and D. Ron. 2002. IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA. *Nature 415:92*.
33. Shapiro-Shelef, M., K.-I. Lin, L. J. McHeyzer-Williams, J. Liao, M. G. McHeyzer-Williams, and K. Calame. 2003. Blimp-1 is required for the formation of immunoglobulin secreting plasma cells and pre-plasma memory B cells. *Immunity 19:607*.
34. Iwakoshi, N. N., A. H. Lee, and L. H. Glimcher. 2003. The X-box binding protein-1 transcription factor is required for plasma cell differentiation and the unfolded protein response. *Immunol. Rev. 194:29*.
35. Dancescu, M., M. Rubio-Trujillo, G. Biron, D. Bron, G. Delespesse, and M. Sarfati. 1992. Interleukin 4 protects chronic lymphocytic leukemic B cells from death by apoptosis and up-regulates Bcl-2 expression. *J. Exp. Med. 176:1319*.
36. Sherr, C. J. 1993. Mammalian G_1 cyclins. *Cell 73:1059*.
37. Glover, D. M., I. M. Hagan, and A. A. Tavares. 1998. Polo-like kinases: a team that plays throughout mitosis. *Genes Dev. 12:3777*.
38. Sherr, C. J., and J. M. Roberts. 1999. CDK inhibitors: positive and negative regulators of G_1-phase progression. *Genes Dev. 13:1501*.
39. Allman, D. M., S. E. Ferguson, and M. P. Cancro. 1992. Peripheral B cell maturation. I. Immature peripheral B cells in adults are heat-stable antigenhi and exhibit unique signaling characteristics. *J. Immunol. 149:2533*.
40. Oliver, A. M., F. Martin, G. L. Gartland, R. H. Carter, and J. F. Kearney. 1997. Marginal zone B cells exhibit unique activation, proliferative and immunoglobulin secretory responses. *Eur. J. Immunol. 27:2366*.
41. Su, T. T., and D. J. Rawlings. 2002. Transitional B lymphocyte subsets operate as distinct checkpoints in murine splenic B cell development. *J. Immunol. 168:2101*.
42. Carsetti, R., G. Kohler, and M. C. Lamers. 1995. Transitional B cells are the target of negative selection in the B cell compartment. *J. Exp. Med. 181:2129*.
43. Donahue, A. C., and D. A. Fruman. 2003. Proliferation and survival of activated B cells requires sustained antigen receptor engagement and phosphoinositide 3-kinase activation. *J. Immunol. 170:5851*.
44. Fruman, D. A., S. B. Snapper, C. M. Yballe, L. Davidson, J. Y. Yu, F. W. Alt, and L. C. Cantley. 1999. Impaired B cell development and proliferation in absence of phosphoinositide 3-kinase p85. *Science 283:393*.
45. Anderson, J. S., M. Teutsch, Z. Dong, and H. H. Wortis. 1996. An essential role for tyrosine kinase in the regulation of Bruton's B-cell apoptosis. *Proc. Natl. Acad. Sci. USA 93:10966*.
46. Aderem, A. 1992. The MARCKS brothers: a family of protein kinase C substrates. *Cell 71:713*.
47. Blackshear, P. J. 1993. The MARCKS family of cellular protein kinase C substrates. *J. Biol. Chem. 268:1501*.
48. McLaughlin, S., J. Wang, A. Gambhir, and D. Murray. 2002. PIP(2) and proteins: interactions, organization, and information flow. *Annu. Rev. Biophys. Biomol. Struct. 31:151*.
49. Wang, J., A. Arbuzova, G. Hangyas-Mihalyne, and S. McLaughlin. 2001. The effector domain of myristoylated alanine-rich C kinase substrate binds strongly to phosphatidylinositol 4,5-bisphosphate. *J. Biol. Chem. 276:5012*.
50. Glaser, M., S. Wanaski, C. A. Buser, V. Boguslavsky, W. Rashidzada, A. Morris, M. Rebecchi, S. F. Scarlata, L. W. Runnels, G. D. Prestwich, et al. 1996. Myristoylated alanine-rich C kinase substrate (MARCKS) produces reversible inhibition of phospholipase C by sequestering phosphatidylinositol 4,5-bisphosphate in lateral domains. *J. Biol. Chem. 271:26187*.
51. Nishi, T., and M. Forgac. 2002. The vacuolar (H^+)-ATPases: nature's most versatile proton pumps. *Nat. Rev. Mol. Cell Biol. 3:94*.
52. Worby, C. A., and J. E. Dixon. 2002. Sorting out the cellular functions of sorting nexins. *Nat. Rev. Mol. Cell Biol. 3:919*.
53. Li, X., T. Wang, Z. Zhao, and S. A. Weinman. 2002. The ClC-3 chloride channel promotes acidification of lysosomes in CHO-K1 and Huh-7 cells. *Am. J. Physiol. Cell Physiol. 282:C1483*.
54. Wagle, N. M., J. H. Kim, and S. K. Pierce. 1998. Signaling through the B cell antigen receptor regulates discrete steps in the antigen processing pathway. *Cell. Immunol. 184:1*.
55. Xu, X., B. Press, N. M. Wagle, H. Cho, A. Wandinger-Ness, and S. K. Pierce. 1996. B cell antigen receptor signaling links biochemical changes in the class II peptide-loading compartment to enhanced processing. *Int. Immunol. 8:1867*.
56. Neuberger, M. S. 1997. Antigen receptor signaling gives lymphocytes a long life. *Cell 90:971*.
57. Aluvihare, V. R., A. A. Khamlichi, G. T. Williams, L. Adorini, and M. S. Neuberger. 1997. Acceleration of intracellular targeting of antigen by the B-cell antigen receptor: importance depends on the nature of the antigen-antibody interaction. *EMBO J. 16:3553*.
58. Clark, M. R., D. Massenburg, M. Zhang, and K. Siemasko. 2003. Molecular mechanisms of B cell antigen receptor trafficking. *Ann. NY Acad. Sci. 987:26*.
59. Chuvpilo, S., E. Jankevics, D. Tyrsin, A. Akimzhanov, D. Moroz, M. K. Jha, J. Schulze-Luehrmann, B. Santner-Nanan, E. Feoktistova, T. Konig, et al. 2002. Autoregulation of NFATc1/A expression facilitates effector T cells to escape from rapid apoptosis. *Immunity 16:881*.
60. Tumanov, A., D. Kuprash, M. Lagarkova, S. Grivennikov, K. Abe, A. Shakhov, L. Drutskaya, C. Stewart, A. Chervonsky, and S. Nedospasov. 2002. Distinct role of surface lymphotoxin expressed by B cells in the organization of secondary lymphoid tissues. *Immunity 17:239*.
61. Dadgostar, H., B. Zarnegar, A. Hoffmann, X.-F. Qin, U. Truong, G. Rao, D. Baltimore, and G. Cheng. 2002. Cooperation of multiple signaling pathways in CD40-regulated gene expression in B lymphocytes. *Proc. Natl. Acad. Sci. USA 99:1497*.
62. Caamano, J. H., C. A. Rizzo, S. K. Durham, D. S. Barton, C. Raventos-Suarez, C. M. Snapper, and R. Bravo. 1998. Nuclear factor (NF)-κB2 (p100/p52) is required for normal splenic microarchitecture and B cell-mediated immune responses. *J. Exp. Med. 187:185*.
63. Franzoso, G., L. Carlson, L. Poljak, E. W. Shores, S. Epstein, A. Leonardi, A. Grinberg, T. Tran, T. Scharton-Kersten, M. Anver, et al. 1998. Mice deficient in nuclear factor (NF)-κB/p52 present with defects in humoral responses, germinal center reactions, and splenic microarchitecture. *J. Exp. Med. 187:147*.
64. Dejardin, E., N. M. Droin, M. Delhase, E. Haas, Y. Cao, C. Makris, Z. W. Li, M. Karin, C. F. Ware, and D. R. Green. 2002. The lymphotoxin-β receptor induces different patterns of gene expression via two NF-κB pathways. *Immunity 17:525*.
65. Senftleben, U., Y. Cao, G. Xiao, F. R. Greten, G. Krahn, G. Bonizzi, Y. Chen, Y. Hu, A. Fong, S. C. Sun, and M. Karin. 2001. Activation by IKKα of a second, evolutionary conserved, NF-κB signaling pathway. *Science 293:1495*.
66. Claudio, E., K. Brown, S. Park, H. Wang, and U. Siebenlist. 2002. BAFF-induced NEMO-independent processing of NF-κB2 in maturing B cells. *Nat. Immunol. 3:958*.
67. Coope, H. J., P. G. Atkinson, B. Huhse, M. Belich, J. Janzen, M. J. Holman, G. G. Klaus, L. H. Johnston, and S. C. Ley. 2002. CD40 regulates the processing of NF-κB2 p100 to p52. *EMBO J. 21:5375*.
68. Kayagaki, N., M. Yan, D. Seshasayee, H. Wang, W. Lee, D. M. French, I. S. Grewal, A. G. Cochran, N. C. Gordon, J. Yin, et al. 2002. BAFF/BLyS receptor 3 binds the B cell survival factor BAFF ligand through a discrete surface loop and promotes processing of NF-κB2. *Immunity 17:515*.
69. Takeda, K., T. Kaisho, and S. Akira. 2003. Toll-like receptors. *Annu. Rev. Immunol. 21:335*.
70. Fambrough, D., K. McClure, A. Kazlauskas, and E. S. Lander. 1999. Diverse signaling pathways activated by growth factor receptors induce broadly overlapping, rather than independent, sets of genes. *Cell 97:727*.
71. Pawson, T., and T. M. Saxton. 1999. Signaling networks: do all roads lead to the same genes? *Cell 97:675*.