Cooperation of multiple signaling pathways in CD40-regulated gene expression in B lymphocytes

Hajir Dadgostar*†, Brian Zarnegar*, Alexander Hoffmann‡, Xiao-Feng Qin‡, Uyen Truong§, Govinda Rao§, David Baltimore‡, and Genhong Cheng*¶

*Molecular Biology Institute and †Medical Scientist Training Program, School of Medicine, University of California, Los Angeles, CA 90095; ‡California Institute of Technology, Pasadena, CA 91125; and §Affymetrix, Incorporated, Santa Clara, CA 95051

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CD40/CD40L interaction is essential for multiple biological events in T dependent humoral immune responses, including B cell survival and proliferation, germinal center and memory B cell formation, and antibody isotype switching and affinity maturation. By using high-density microarrays, we examined gene expression in primary mouse B lymphocytes after multiple time points of CD40L stimulation. In addition to genes involved in cell survival and growth, which are also induced by other mitogens such as lipopolysaccharide, CD40L specifically activated genes involved in germinal center formation and T cell costimulatory molecules that facilitate T dependent humoral immunity. Next, by examining the roles of individual CD40-activated signal transduction pathways, we dissected the overall CD40-mediated response into genes independently regulated by the individual pathways or collectively by all pathways. We also found that gene down-regulation is a significant part of the overall response and that the p38 pathway plays an important role in this process, whereas the NF-κB pathway is important for the up-regulation of primary response genes. Our finding of overlapping independent control of gene expression modules by different pathways suggests, in principle, that distinct biological behaviors that depend on distinct gene expression subsets can be manipulated by targeting specific signaling pathways.

CD40 is a member of the pleiotropic tumor necrosis factor receptor (TNFR) superfamily, which also includes such functionally diverse molecules as CD27, CD30, OX40, RANK, LT-βR, the p75 low-affinity nerve growth factor receptor, Fas, and members of the death receptor family as well as TNFR1 and TNFR2 (1). CD40 stimulation activates B cells and promotes various aspects of a functional humoral immune response, including enhancement of survival and proliferation (2). Like many TNFR family members, CD40 activates the JNK/SAPK and NF-κB pathways (3, 4). Both of these pathways involve serine/threonine kinases that regulate gene expression through activation of AP1 and Rel transcription factors, respectively. Another stress-responsive pathway that has also been reported to be activated by CD40 (5) is the p38 kinase pathway, which leads to the phosphorylation and activation of transcription factors such as ATF2 (6). Previous work has also linked CD40 to the activation of the extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) pathway (7).

In addition to serine/threonine kinases, a link between CD40 signaling and tyrosine kinase activation has been suggested (8). One pathway shown to be activated by CD40 and to play an important role in CD40 signaling is that initiated by PI-3 kinase (PI-3K) (8–10). PI-3K phosphorylates membrane phospholipids (11), which can recruit and activate pleckstrin homology domain-containing molecules such as the kinase protein kinase B. This kinase may then regulate the activity of forkhead-related transcription factors (12, 13).

In each of the above pathways, one of the major final outcomes is an alteration in the activity of one or more transcription factors, and hence it is generally believed that CD40 achieves many of its complex effects on B cells through alterations in gene expression. CD40 up-regulates expression of numerous genes, including CD23, intercellular adhesion molecule-1 (ICAM-1), Fas, B7.1, B7.2, MHC II, LT-α, c-*myc*, Bcl-x, Bfl-1, A20, CDK4, CDK6, IgCγ, and IgCε (2, 14–21). From the standpoint of signaling specificity, it is not presently clear which (if any) CD40-mediated gene expression responses are specific to CD40, as a B cell costimulus, and which are less specific manifestations of mitogenic B cell activation. Also, the nature of the cooperation between different CD40-mediated signaling pathways in the overall gene expression response has not been defined. For this purpose, gene expression profiling can be used to measure the relative impact of each pathway in CD40-mediated B cell activation and to examine the nature of that impact. With these goals in mind, we have undertaken a controlled and systematic screen for CD40-mediated gene expression in B cells and have dissected the contributions of multiple CD40-activated signaling pathways to early gene expression through the use of high-density oligonucleotide microarrays.

Materials and Methods

Primary B Cell Isolation and Culture. Spleens were isolated from 8- to 10-week-old C57BL/6J mice (The Jackson Laboratory). Splenic T cells were removed by incubating with monoclonal anti-Thy1.2 IgM (Sigma) followed by lysis with rabbit complement (Cedarlane Laboratories). At this step, the cells were typically 95% pure B220$^+$ B cells by FACS analysis. To obtain highly pure naïve B cells for microarray studies, these cells were stained with a biotin-conjugated anti-CD43 antibody (PharMingen) followed by streptavidin-conjugated magnetic microbeads (Miltenyi Biotec, Auburn, CA) and passed through a depletion-type magnetic sorting column (Miltenyi Biotec). Unbound cells were collected as the purified resting B cell sample—over 98% pure B220$^+$ B cells expressing low levels of activation markers such as ICAM-1, CD23, and B7.2 by FACS analysis (data not shown). B cells were cultured in RPMI media 1640 supplemented with 10% FBS, 50 μM β-mercaptoethanol, and 1% penicillin/streptomycin (Life Technologies, Rockville, MD) at 37°C under 10% CO_2. Cells were stimulated with soluble CD8/CD40L fusion at 300 ng/ml or lipopolysaccharide at 20

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Abbreviations: MAPK, mitogen-activated protein kinase; PI-3K, PI-3 kinase; ICAM-1, intercellular adhesion molecule-1; CHX, cycloheximide; ERK, extracellular signal-regulated kinase.

¶To whom reprint requests should be addressed at: Department of Microbiology and Molecular Genetics, Jonsson Comprehensive Cancer Center, and Molecular Biology Institute, University of California, Los Angeles, CA 90095. E-mail: genhongc@microbio.ucla.edu.

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μg/ml unless otherwise indicated. Cells stimulated for microarray analysis were cultured at 2.5×10^6 cells/ml.

Measurement of Cellular Proliferation and Apoptosis. For proliferation assays, B cells were stimulated at 5.0×10^5 cells/ml in 96-well plates for 42 h, pulsed with 0.5 μCi ^{3}H-thymidine per well, incubated for an additional 6 h, and collected on a 96-well filtermat by using an automated harvester. Activity was measured in a 96-well format on a scintillation counter. Cell death was measured through FACS analysis after 24 h of culture by double staining with propidium iodide and FITC-conjugated anti-Annexin V antibody. The percentage of cells in the double-negative quadrant was recorded as the viable fraction.

Target Preparation and Microarray Analysis. Primary B cells were flash frozen in liquid nitrogen after stimulation for 2 h with or without soluble CD40L in the presence of DMSO (0.1%), U0126 (10 μM), SB203580 (10 μM), or LY294002 (10 μM), or for variable lengths of time in the presence of CD40L (300 ng/ml) alone or lipopolysaccharide (20 μg/ml) alone or in media. Total and poly(A)$^+$ RNA were isolated by using commercially available kits (Qiagen, Chatsworth, CA) with a typical yield of 5–10 μg total and 0.5–1.0 μg of poly(A)$^+$ RNA per condition. Synthesis of cDNA was carried out by using a T7-oligo-dT primer (Gensent, La Jolla, CA) with Superscript-II RT and related reagents (GIBCO). *In vitro* transcription of biotin-labeled cRNA was carried out by using a commercially available kit (Enzo Diagnostics). Yield of cRNA typically ranged from 40 to 80 μg per condition.

Mu6500 expression probe arrays (Affymetrix) were used for expression screening. Hybridization was carried out at 45°C for 16 h under constant rotation. After washing and staining, chips were scanned by using an argon–ion laser scanner (Hewlett–Packard) at a wavelength of 570 nM. Gene expression results were captured, normalized through global scaling, and analyzed by using GENECHIP analysis software (Affymetrix).

Hierarchical Clustering. Determination of gene induction, inhibition, and pathway dependence was based on multiple GENECHIP parameters, including fold change and average difference change, as explained in detail in the supporting information on the PNAS web site, www.pnas.org. Hierarchical clustering was performed by using publicly available software (http://rana.stanford.edu/software/). Single-axis clustering of average difference change values (Fig. 2) and bidirectional clustering of pathway dependence profiles (Fig. 3) were performed through average linkage clustering by using an uncentered Pearson's correlation coefficient as the similarity metric.

Results

The Roles of Multiple Pathways in CD40-Mediated Survival and Proliferation. Survival and proliferation are two well-known responses of B cells to CD40 activation. Much remains to be learned, however, about the specific signaling pathways and downstream gene expression responses responsible for such fundamental biological effects. As a functional measure of CD40 signaling in B cells, we examined the ability of CD40L to protect cultured primary B cells from spontaneous apoptosis and to promote proliferation in the presence of different pathway inhibitors. As shown in Fig. 1*A*, CD40 continues to promote survival in B cells in the presence of pharmacological inhibitors of the ERK, p38, or PI-3K pathways. Although blocking ERK activation or PI-3K function does produce some reduction in the viable percentage, there is still a significant response to CD40 stimulation compared with baseline.

In contrast to the survival response, B cell proliferation is extremely sensitive to all of the conditions examined. As shown in Fig. 1*B*, each pathway inhibitor diminishes the proliferative

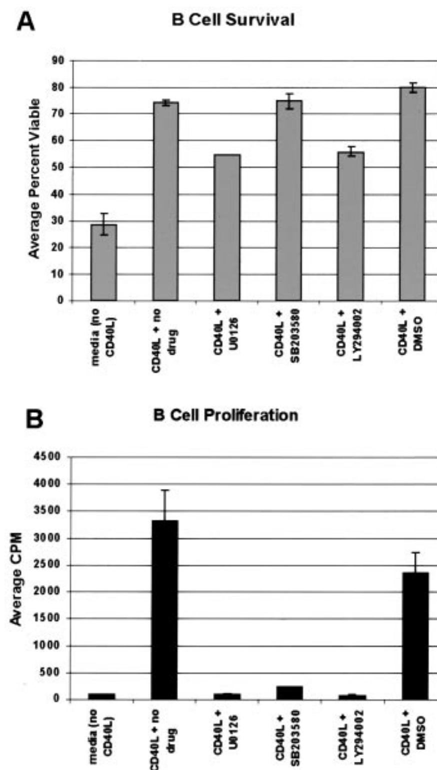

Fig. 1. Contribution of multiple pathways is required for CD40-mediated proliferation but not survival signaling. (*A*) Charts display the percentage of viable B cells (as measured by FACS analysis after propidium iodide-annexin V staining) after CD40L stimulation with or without various pharmacological inhibitors. (*B*) Charts display the level of proliferation of CD40L-stimulated B cells (as measured by incorporation of ^{3}H-labeled thymidine) with or without various pharmacological inhibitors.

response down to near-basal levels. Because proliferation is a complex process requiring the cooperation of multiple functional classes of genes at many levels, it is likely to be sensitive to biochemical perturbations within any one of numerous signaling pathways.

Genes with Roles in Adhesion, Migration, and Lymphoid Organization Are Induced in a CD40-Specific Manner. To examine the evolution of B cell responses to CD40 stimulation over an extended time course, we stimulated primary B cells with soluble CD40L for 4, 12, 24, or 72 h or 1 week in culture. We then examined gene expression profiles by using microarrays representing 6,350 murine sequences. Clustering was performed to distinguish genes based on temporal expression patterns (see supporting information on the PNAS web site).

Because many of the effects of CD40 stimulation on B cells may be common to a number of mitogenic stimuli, we differentiated CD40-specific responses from general mitogenic effects through a comparative analysis by using another potent B cell mitogen, lipopolysaccharide, as a control. As summarized in Fig. 2, many of the CD40-specific genes identified in this fashion were genes with roles in cellular adhesion, migration, and lymphoid organization. Both lymphotoxin-α and -β, which together play an indispensable role in dendritic cell recruitment and germinal center formation (22, 23), were induced specifically by CD40. Other CD40-induced genes with potentially important roles in cellular recruitment and interaction within sites of B cell activation are the receptor for macrophage inflammatory protein-1α and properdin. The latter is a C3-convertase stabilizer that functions in the activation of the alternative complement path-

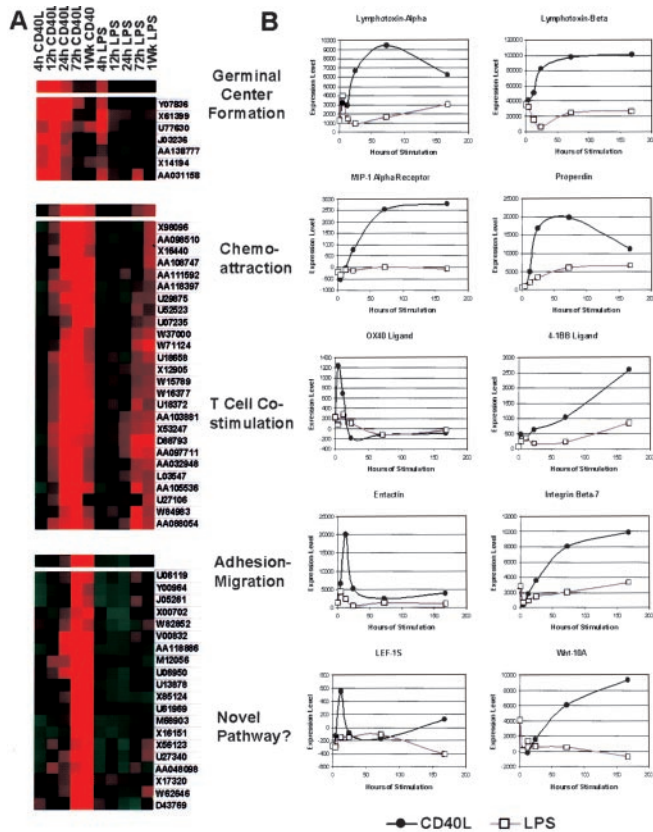

Fig. 2. CD40-specific genes include numerous mediators of lymphoid trafficking and communication. (*A*) Hierarchical clustering was performed to distinguish stimulus-specific gene clusters. Shown are representative early (*Top*) and middle and late (*Bottom*) CD40-specific clusters. (*B*) Examples of CD40-specific genes with potential roles in immune cell trafficking and communication.

way, potentially leading to localized release of chemotactic complement products. In addition, CD40 induces the expression of OX40 ligand and 4–1BB ligand, two members of the tumor necrosis factor family with important roles in the activation of T cells and in their recruitment to lymphoid follicles (24, 25). Also included in Fig. 2 are integrin β-7 and the extracellular matrix protein entactin. The role of integrins in leukocyte adhesion and homing is well established. Entactin is known to play important roles in adhesion and migration in other systems (26), although its role in B cell biology has not been established. Interestingly, the CD40-specific induction of Wnt-10a, a recently characterized member of the Wnt family, LEF-1S, a transcriptional mediator of Wnt-activated responses and the Disheveled signaling molecule (not shown), suggests a novel potential connection between CD40-mediated B cell activation and Wnt signaling.

Multipathway Dissection of the CD40-Regulated Expression Response. We next examined the contributions of the PI-3 kinase, p38 kinase, and ERK pathways to early CD40-mediated gene regulation by using pharmacological inhibitors to block each signal individually during stimulation. Because there are five NF-κB subunits with roles in transcriptional regulation, mice with a complete defect in all subunits are not currently available. Thus, we examined CD40-regulated gene expression in B cells from mice bearing homozygous mutations in both the p50 and c-Rel genes—both of which play important roles in lymphocyte activation (27, 28)—and a heterozygous mutation at the p65 locus. These compound mutants, NF-κB-2.5 knockout mice, represent the most severe defect in lymphocyte-mediated NF-κB signaling currently available in a viable animal.

To distinguish primary responses from secondary ones (i.e., those that depend on new protein synthesis), stimulation was also performed in the presence of the protein synthesis inhibitor cycloheximide (CHX). We found that 30 of 86 CD40L-induced genes were induced in the presence of CHX, and 52 of 128 CD40L-inhibited genes were inhibited in the presence of CHX (see supporting information on the PNAS web site). Many of the protein synthesis-independent gene induction responses are genes previously reported to be targets of NF-κB signaling, including Bcl-x, A1, TRAF1, ICAM-1, A20, c-myc, IL-1β, and IκB-α (16, 21, 29, 30), which suggests that this pathway dominates the primary response to CD40L. Indeed, many of the other CHX-insensitive induced genes, such as the cytokine EBI3, the putative G protein-coupled receptor TDAG8, and the adhesion receptor CD83, may also prove to be regulated by the NF-κB pathway.

Over half of the genes inhibited by CD40 stimulation appear to require new protein synthesis for their down-regulation. The down-regulation of many transcription factors (e.g., IRF2, NIP45, and Fli-1), potential growth repressors (SPA-1, Rb2/p130), and kinases involved in MAPK pathways, however, appears CHX-insensitive. Thus, the number of genes regulated and the percentage of those genes likely to represent primary responses are similar for CD40-induced and -inhibited genes.

The results from all pathways tested were then pooled together and hierarchically clustered, and in Fig. 3, we focus on representative clusters of CD40-induced and -inhibited genes. Among both CD40-induced and -inhibited genes, there are groups of genes whose regulation does not depend on any of the pathways tested (core response genes). A large fraction of the genes display the opposite behavior in terms of pathway dependence—they depend on most or all pathways tested (collective control genes). Finally, there are a number of genes whose regulation depends on a single pathway or a combination of two pathways (independent control genes).

NF-κB-2.5 Knockout. The induction of a number of genes was found to be sensitive to this partial NF-κB deficiency. As expected, many of these genes are also CHX-insensitive; however, many of the primary response genes previously associated with the NF-κB pathway (e.g., Bcl-x, A1, ICAM-1, A20, etc.) are not affected. Indeed, the effect of this compound mutation on CD40-mediated B cell activation was far less severe than initially expected, and possible reasons for this are discussed below. Although this is not a complete mutation of all NF-κB family members, these results do indicate that many of the most highly induced CD40-responsive genes in B cells do not require either p50 or c-Rel for their induction.

By the same token, the NF-κB targets c-myc and IL-1β and many other CD40-induced genes were found to require one or both of these subunits (or perhaps a higher dose of the p65 gene) for their induction. These genes included the primary response gene EBI3, a cytokine homologous to the p40 subunit of IL-12 (31). They also included TDAG8, a putative G protein-coupled receptor in the same family as G2A, a recently cloned receptor with a potential role in cell cycle arrest (32). Also affected were the potential secondary response genes complement receptor 2, STAT-5a, and the mysterious nuclear molecule Jumonji, which plays an important role in development and may be a growth inhibitor (33).

ERK Pathway. In addition to clustering genes according to their pathway dependence, individual treatment conditions were also clustered according to the genes whose regulation they affected. In general, most gene regulation is not affected by U0126, suggesting that early CD40-mediated responses are largely in-

Fig. 3. Differential requirements for various CD40-activated signaling pathways in gene induction and inhibition. Dependence of gene induction/inhibition on multiple pathways and on new protein synthesis (CHX) was determined as described in *Materials and Methods*, and the results were hierarchically clustered. Inhibited pathways: p50−/−:c-Rel−/−:p65−/+ (κB 2.5-KO), ERK (U0126), p38 (SB203580), and PI-3K (LY294002). Closeups of representative clusters of (*A*) induced and (*B*) inhibited genes are shown. Gold squares represent genes whose induction/inhibition by CD40L is independent of a given pathway (e.g., not blocked by addition of drug). Blue squares represent genes that depend on a given pathway, and black squares represent genes that did not meet criteria for classification as either independent or dependent.

dependent of the ERK pathway. For this reason, the U0126 condition forms an outgroup in the tree shown in Fig. 3*A*. Indeed, when clustering is performed according to overall gene expression patterns among the different arrays tested, expression profiles from CD40L-treated cells in the presence of U0126 are closest to those from control CD40L-treated cells (data not shown).

PI-3K Pathway. When clustering according to induced genes, LY294002 (a PI-3K blocker), and CHX are grouped together. As demonstrated in Fig. 3*A*, this is largely because many of the independent control genes corresponding to the PI-3K pathway are also CHX-sensitive. These include various transporters and enzymes involved in metabolic processes (e.g., monocarboxylate transporter, reduced folate carrier, Ca^{2+} ATPase 2, alkaline phosphatase, sialyltransferase 10, etc.). This result suggests one of the roles of this pathway in CD40 biology may be up-regulation of metabolic mediators needed by the activated and growing cell. Roughly balancing the role of PI-3K in gene induction is its role in gene inhibition. It is required for the down-regulation of transcription factors, such as LKLF, TSC-22, N-Oct3, NIP45, ZFP-90, and IRF-2, as well as adhesion receptors such as L-selectin and integrin-α6.

p38 Pathway. Clustering of pathways based on down-regulated genes produces a different arrangement. The major outgroup in Fig. 3*B* is SB203580 (a p38 inhibitor), because this pathway appears to play the most prominent role in gene inhibition. The p38 pathway is distinguished from the other pathways in this regard by a group of independent control genes, almost all CHX-insensitive, whose down-regulation is prevented only when the p38 pathway is blocked. The majority of all primary CD40-inhibited genes require this pathway for their down-regulation These include, among other genes, a number of detoxification/stress response genes such as catalase, glutathione peroxidase, and aldehyde dehydrogenase. Also included are several MAPK pathway components such as ERK, MEKK3, and p38 itself, possibly representing a mechanism for collective negative feedback on MAPK pathways.

Discussion

Through a systematic screen for CD40-regulated gene expression, we identified a large number of previously unrecognized CD40 targets with various biological roles. These are primary as well as secondary response genes and include transcription factors, cytokines, receptors, signaling molecules, proapoptotic molecules, regulators of proliferation, and metabolic mediators.

Many CD40-specific genes represent potential mediators of germinal center formation in T dependent immune responses, distinguishing CD40 from lipopolysaccharide, a T independent activator. It is within germinal centers that events critical to affinity maturation and memory, two hallmarks of T dependent immunity, take place. CD40 and CD40L are required for the formation of these lymphoid structures (34). Furthermore, the recruitment of follicular dendritic cells (a prerequisite for the formation of germinal centers) depends on B cells. Lymphotoxin expression is instrumental in this role of B cells (22, 23). We find that both LT-α and -β (together forming the functional ligand for LT-βR) are induced in a CD40-specific manner. In addition, a number of other candidate molecules with roles in chemoattraction, cellular adhesion, and migration are induced by CD40. These molecules also may play roles in various steps of this complex process. Our data suggest that a distinct signal activated only by CD40 is required for the induction of lymphotoxins and other candidate germinal center-promoting genes. In support of this notion, it was recently demonstrated *in vivo* that LMP1, an Epstein–Barr virus-encoded protein thought to signal through similar pathways, can rescue some but not all CD40-mediated functions in CD40−/− animals (35). Specifically, it was found that germinal center formation was not rescued by LMP1.

A large number of mRNAs are decreased after CD40 stimulation. Other studies comparing the gene expression responses of

B cells to tolerizing vs. activating antigen found that foreign stimulation led to a similar down-regulation profile (36). The large number of transcriptional regulators (18 total) that are down-regulated, many of them transcriptional repressors, suggests that gene inhibition contributes to CD40-regulated transcriptional reprogramming, for instance, by releasing other genes from transcriptional repression. Several genes whose expression in cells has been shown to lead to cell cycle arrest, including Rb-1, Rb-2, SLAP, BTG-2, and SPA-1 (37–40), were also down-regulated. This may also be a mechanism for releasing resting B cells from quiescence. Finally, a number of kinases associated with B cell activation (e.g., p38, ERK, and BTK) were also down-regulated, suggesting a mechanism for feedback regulation of such pathways.

Among induced genes, the core response group includes a number of previously characterized NF-κB targets, including ICAM-1, TRAF1, Bcl-x, A1, and A20 (16, 21, 29, 30). The compound mutant animals used to test the role of this pathway (p50−/−, c-Rel−/−, p65+/−) are not completely deficient in NF-κB activity. This result indicates that in the induction of these genes, but not in the induction of genes such as *c-myc*, *IL-1β*, *TDAG8*, and *EBI3*, other NF-κB subunits or other pathways are able to compensate. In support of the view that most of these core response genes are indeed NF-κB targets, we have found that the induction of these genes is blocked in the presence of molecules such as gliotoxin and SN-50, which block all NF-κB activity (data not shown).

Like the NF-κB pathway, the PI-3K pathway makes a major contribution to gene induction by CD40. Although many of the genes that depend on NF-κB were primary response genes, the majority of PI-3K-dependent genes are secondary response genes. These include a number of metabolic enzymes and transporters. Although previous work has reported a link between PI-3K and the NF-κB pathway (41–43), we find in our system that inhibition of PI-3K does not interfere with the induction of many NF-κB-regulated genes. In addition, we find that in primary B cells, inhibiting PI-3K through the use of LY294002 does not reduce CD40-mediated NF-κB activation as measured by electrophoretic mobility-shift assay (data not shown).

The role of the p38 pathway in gene induction appears to be smaller than that of the other two pathways and almost entirely cooperative (i.e., also requiring other pathways). Interestingly, however, this pathway appears to play a major role in CD40-mediated gene down-regulation. A significant portion of this role appears to be independent (i.e., not involving any of the other pathways tested) and primary in nature. Over half of the genes inhibited in a CHX-insensitive manner require this pathway, and many of the rest were core response genes not affected by any of the pathways tested. The mechanism through which p38 achieves this effect is of great interest as it represents a potentially novel mechanism contributing to CD40 biology. One mechanism may be through transcriptional repression. Although the mediator(s) of such repression is unclear, studies on the regulation of insulin, cyclin D2, and retinoid-X receptor (44–46) have revealed enhanced expression on inhibition of p38 signaling. Another mechanism may be related to RNA stability, although a role for p38 in destabilizing specific messages has not been established. In fact, current evidence from studies on cyclooxygenase-2 and various cytokines indicates a positive role for p38 in RNA stabilization (47–49). Finally, the p38 pathway has also been examined as an inhibitor of other MAPK pathways (50). Thus, the inhibitory role of p38 may be primarily at the signaling level, perhaps through activation of a downstream phosphatase.

Among the pathways tested, the ERK pathway appears to have a very minor role in early CD40-mediated gene expression. We know that this pathway does play a role in CD40-mediated B cell activation because the dose of U0126 used leads to complete inhibition of CD40-mediated ERK phosphorylation (data not shown) and also completely inhibits CD40-induced B cell proliferation (Fig. 1*B*). Although some suggest that ERK is activated downstream of CD40 in B cells (7), others have suggested that this may not be the case (4, 5) or that CD40-induced ERK activation may be more prominent in monocytic cells than in B cells (51). Our results, based on early gene expression, agree with the latter hypothesis.

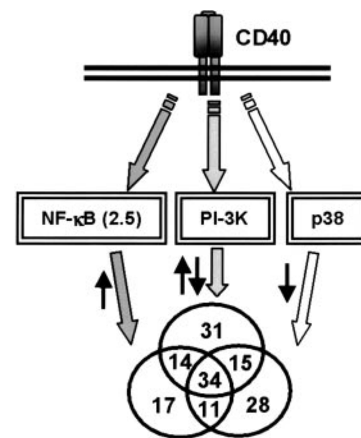

Fig. 4. Toward a model of B cell activation through CD40-regulated gene expression. Partially overlapping dependence of CD40-regulated gene subsets (represented by numbers of pathway-dependent genes in the Venn diagram) on three major pathways. Small arrows indicate the major contributions of different pathways to gene up- and/or down-regulation. The overall gene regulation response to CD40 stimulation consists of a number of partially overlapping gene expression subsets, each depending on a particular pathway. The partially overlapping nature of these subsets suggests a balance between independent control (i.e., distinct gene subsets being regulated by different pathways) and collective control (i.e., a subset of genes whose regulation depends on all pathways tested). Thus, all pathways contribute to the regulation of a single group of genes (the collective control cluster), whereas each pathway also regulates a distinct subset of genes in its independent control cluster.

Inhibiting each of these pathways has a profound effect on CD40-mediated proliferative responses but a much less severe effect, if any, on CD40-mediated B cell survival. One reason for this result may be that survival is a less complex response, requiring the induction of fewer genes. Accordingly, nearly all CD40-regulated genes with proposed roles in cell death regulation, such as Bcl-x, A1, and A20, are core response genes with regulation profiles independent of the pathways examined (Fig. 3*A*). On the other hand, proliferation is most likely a more complex response requiring the cooperation of multiple pathways. For instance, the NF-κB pathway regulates the induction of the oncogenic transcription factor c-myc, which has a documented role in cellular proliferation (52). The p38 pathway may contribute by down-regulating the expression of genes, such as Ndr1, Rb2, and SPA-1 (Fig. 3*B*), whose expression may negatively regulate growth (37, 38). PI-3K may play a similar role by down-regulating Rb1 as well as BTG-2, which mediates antiproliferative activity through Rb-dependent mechanisms (40). PI-3K most likely also contributes as a key regulator of a number of metabolic enzymes and transporters (Fig. 3).

There are three hypothetical mechanisms to account for the regulation via gene expression of complex biological responses by multiple signaling pathways downstream of a single receptor. We call these the independent control, collective control, and redundant control scenarios for multipathway gene regulation. In the independent control scenario, each pathway contributes to the overall gene expression response by regulating a distinct

subset of genes. These different gene subsets then combine to make up the overall gene expression profile of the stimulus and cooperatively give rise to its biological effects. In the collective control scenario, a number of signal transduction pathways cooperate to regulate a single large set of genes that constitute the entire gene expression profile. This cooperation may occur either at the signaling level, via interpathway crosstalk, or at the transcriptional level, through multicomponent transcriptional complexes. Finally, in the redundant control situation, different pathways can substitute for one another in the regulation of each downstream gene. Thus, the overall expression response remains intact in the absence of a single pathway. Other work examining the roles of multiple pathways in growth factor-stimulated fibroblasts has suggested a high degree of functional redundancy among pathways, as most gene induction was either unaffected or broadly affected by different manipulations (i.e., redundant vs. collective control) (53). We find, as shown in Fig. 3, that there are some subsets whose regulation fits the independent control mechanism and other genes that appear to require all or most of the pathways tested for their regulation (i.e., collective control). Thus, both the independent and collective mechanisms describe the response of primary B cells to CD40 activation (Fig. 4).

In summary, we define a mechanism for the cooperation of multiple signaling pathways in regulating overall early gene expression downstream of CD40. The NF-κB pathway contributes to primary gene induction, the PI-3K pathway contributes to gene induction and inhibition, and the p38 pathway contributes primarily to gene inhibition. The ERK pathway appears to have little role in early CD40-mediated gene regulation. In addition, CD40-regulated genes were classified into one of three categories reflecting their pathway-dependence characteristics: core response, independent control, and collective control. The distribution of genes among these categories was roughly balanced, indicating that both independent and collective control play significant roles in the response of B cells to CD40, suggesting, in principle, that individual gene subsets downstream of CD40 can be targeted by manipulating the CD40-mediated activation of a single signal transduction pathway while leaving other responses intact.

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