Unique CD40-mediated biological program in B cell activation requires both type 1 and type 2 NF-κB activation pathways

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B lymphocytes can be activated by many different stimuli. However, the mechanisms responsible for the signaling and functional specificities of individual stimuli remain to be elucidated. Here, we have compared the contribution of the type 1 (p50-dependent) and type 2 (p52-dependent) NF-κB activation pathways to cell survival, proliferation, homotypic aggregation, and specific gene regulation of murine primary B lymphocytes. Whereas lipopolysaccharide (LPS) and B cell activation factor (BAFF) mainly activate the type 1 or type 2 pathways, respectively, CD40 ligand (CD40L) strongly activates both. Rescue of spontaneous apoptosis is diminished in $p52^{-/-}$ B cells after BAFF stimulation and in $p50^{-/-}$c-$Rel^{-/-}$ B cells after LPS stimulation. Interestingly, significant CD40-induced B cell survival is still observed even in $p50^{-/-}$c-$Rel^{-/-}p65^{-/+}$ B cells, which is correlated with the ability of CD40L to up-regulate Bcl-x_L expression in these cells. CD40L- and LPS-induced B cell proliferation, as well as up-regulation of proliferation-related genes, however, are greatly reduced in c-$Rel^{-/-}$ and $p50^{-/-}$c-$Rel^{-/-}$ B cells but are normal in $p52^{-/-}$ B cells. We have further demonstrated that both c-Rel and p52 are required for CD40-mediated B cell homotypic aggregation, which explains well why neither LPS nor BAFF has this function. Overall, our studies suggest that both type 1 and type 2 NF-κB pathways contribute to the gene expression and biological program unique for CD40 in B cell activation.

B lymphocytes must integrate multiple biochemical signaling pathways to propagate humoral immune responses. Throughout this process, B cells are activated by a number of stimuli with overlapping and unique biochemical pathway activation potential. The unique contribution of individual receptors participating in B cell-mediated immune responses continues to be of great interest. CD40, a member of the tumor necrosis factor receptor family, plays a central role in humoral immunity (1). CD40 activation of B cells results in the activation of numerous biochemical pathways, contributing to biological outcomes including enhanced cell survival and proliferation, germinal center formation, memory B cell development, and Ig isotype switching and affinity maturation (2). Although we know the contribution of multiple biochemical signaling pathways to each of these processes, the mechanisms of CD40's unique contributions are still not clear.

CD40 ligation, in common with a number of B cell stimuli, activates the Rel/NF-κB transcription factors, which are major regulators of immune and inflammatory responses (3, 4). Five members of this family exist in mammals: NF-κB1 (encoding p105, which is constitutively processed to p50), NF-κB2 (encoding p100, which is processed to p52), RelA (p65), c-Rel, and RelB. In unstimulated cells, inactive NF-κB dimers reside in the cytoplasm through interaction with one of a family of inhibitory molecules, collectively termed inhibitors of κB (IκBs). Appearance in the nucleus of NF-κB capable of DNA binding and transcription enhancement results from the activation of kinases that phosphorylate key serine residues on IκBs, signaling their ubiquitination and degradation (5). Recent work has shown two distinct pathways regulating phosphorylation of IκB proteins, resulting in the release of specific and distinct Rel dimers. Type 1 involves activation of the IKK complex, which consists of two catalytic subunits (IKKα and IKKβ) and one regulatory subunit (IKKγ/NEMO). Activation of the IKK complex results in the degradation of IκBα and -β, which release p50:RelA and p50:cRel dimers (6–9). Type 2 involves activation of NF-κB-inducing kinase, which associates with two IKKα molecules. Together, they recognize and bind to the C-terminal portion of p100 (also referred to as IκBδ), resulting in p100 processing to p52 and preferential release of p52:RelB dimers (10, 11). In recent years, the generation of gene knockout mice has helped to elucidate the contribution of Rel family members to a diverse set of cellular responses. Although these studies have revealed overlapping contributions of Rel proteins to processes such as proliferation and cell survival, they have also identified distinct biological roles of individual family members (12–16). As such, differential activation of the type 1 and type 2 NF-κB pathways has potentially important implications for receptor-mediated gene induction.

Previous work examined gene expression profiles in B cells activated by soluble CD40 ligand (sCD40L) and lipopolysaccharide (LPS) (17). We found that the two stimuli activated similar sets of biochemical pathways but that each also activated a unique gene set. Recent studies defining the contribution of p52 and RelB have shed light on the tremendous contribution of the type 2 NF-κB pathway to humoral immunity (15, 18). This finding led to our speculation that the unique gene set induced by sCD40L, which included genes involved in adhesion, migration, lymphoid organization, and germinal center formation, could result from CD40L activation of the type 2 NF-κB pathway, a property that LPS lacks.

In the present study, we analyzed the contribution of type 1 and type 2 NF-κB activity to CD40-mediated responses in primary B cells. Through use of mice deficient in one or more Rel family member, we show that the type 1 and type 2 NF-κB pathways differentially contribute to CD40-mediated B cell survival, proliferation, adhesion, and gene expression. We show that cellular responses common to CD40L and LPS treatment of B cells require their shared capacity to activate the type 1 NF-κB pathway. In addition, we show that other B cell responses, unique to CD40, result from the receptor's ability to activate both arms of the NF-κB pathway.

Materials and Methods

Primary B Cell Isolation and Culture. Spleens were isolated from 8- to 10-week-old $p50^{-/-}$, $p50^{-/-}$c-$Rel^{-/-}$, $p50^{-/-}$c-$Rel^{-/-}p65^{-/+}$,

Abbreviations: LPS, lipopolysaccharide; BAFF, B cell activation factor; hBAFF, human BAFF; BAFF-R, BAFF receptor; CD40L, CD40 ligand; sCD40L, soluble CD40L; Q-PCR, quantitative real-time PCR; IκB, inhibitor of κB; IC, IMAGE clone; TLR, Toll-like receptor.

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and C57BL/6J WT control mice or from 129/SvPasIcoCrlBR WT mice, which served as controls for spleens isolated from c-Rel$^{-/-}$ and p52$^{-/-}$ mice. To obtain highly pure naïve B cells, total splenocytes were stained with a biotin-conjugated anti-CD43 Ab (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: >96% pure B220^{+} B cells expressing low levels of activation markers such as intercellular adhesion molecule 1, CD23, and B7.2 by fluorescence-activated cell sorter analysis (data not shown). B cells were cultured in RPMI medium 1640 supplemented with 10% FBS, 50 μM 2-mercaptoethanol, and 1% penicillin/streptomycin (Life Technologies, Rockville, MD) at 37°C under 10% CO_2. Cells were stimulated (unless indicated otherwise) with 15 μg/ml anti-CD40 mAb, FgK-45, 5 μg/ml LPS, or 100 ng/ml human B cell activation factor (hBAFF) (Amgen Biologicals).

Measurement of Cellular Proliferation and Apoptosis. For proliferation assays, B cells were stimulated at 1.0×10^6 cells per ml in 96-well plates for 48 h, pulsed with 0.5 μCi (1 Ci = 37 GBq) of [^{3}H]thymidine per well beginning 20 h poststimulation, and collected on a 96-well filtermat with an automated harvester. Activity was measured in a 96-well format on a scintillation counter. For viability assays, B cells were stimulated as above and cell death was measured through fluorescence-activated cell sorter analysis after 48 h of culture by double staining with propidium iodide and FITC-conjugated anti-annexin V Ab. The percentage of cells in the double-negative quadrant was recorded as the viable fraction.

RNA Quantitation. RNA was isolated for Northern blot and quantitative real-time PCR (Q-PCR) by using TRIzol (Invitrogen) or standard guanidinium isothiocyanate methods, respectively. Northern blotting was done as described in ref. 19. cDNA fragments purchased from Research Genetics (Huntsville, AL) were used as probes for the detection of *c-Myc* [IMAGE clone (IC) 3663016], *CDK4* (IC 477933), *cyclin D2* (IC 2064983), *AID* (IC 3333637), and *LEF-1* (IC 1446606). Probes cloned via PCR used the following oligonucleotides amplified from a B cell cDNA library with the indicated primers: *Entactin*, 5′-TGGAACGAGAGGACATCCTTG (forward) and 5′-TGCCTCTGGGATTCACCAA (reverse); *Bcl-x$_L$*, 5′-ATGTCTCAGAGCAACCGGGA (forward) and 5′-TCACTTCCGACTGAAGAGTG (reverse); and *UNG*, 5′-GGGAAGCCGTACTTCGTCAA (forward) and 5′-GATGGGCTTCTTGCCAGACTT (reverse). For Q-PCR analysis, 1 μg of RNA was reverse-transcribed by using SuperScript II (Invitrogen) according to the manufacturer's instructions with oligo(dT) and random hexamers serving as primers. Q-PCR analysis was done with the iCycler Thermocycler and Optical System (Bio-Rad) as described in ref. 20. The primers listed here were used to amplify a specific 85- to 100-bp fragment of the following genes: *c-Myc*, 5′CTGCGACGAGAAGAGAGAATTTC (forward) and 5′-ACGCTGAATTTCTTCCAGATATCC (reverse); *cyclin D2*, 5′-CCGCCAGGAGCAAATCGAA (forward) and 5′-GACTTGGATCCGGCGTTATG (reverse).

Immunoblotting. Whole-cell lysate and cell fractionation and nuclear Western blotting were done as described in ref. 19. B cells were lysed in radioimmunoprecipitation assay buffer or fractionation buffer, and protein was quantified by using the BCA Protein Quantification Kit (Pierce). Thirty micrograms of protein was added to each lane and separated by either 9% or 12% SDS/PAGE. Abs specific for murine Bcl-x$_L$, p65, p52, and upstream stimulatory factor 2 were obtained from Santa Cruz Biotechnology. Anti-actin Ab and nonspecific rabbit IgG were purchased from Sigma. Gels were transferred to nitrocellulose membranes and immunoblotted according to the Ab manufacturer's recommended instructions.

Flow Cytometry of Intracellular Proteins. For intracellular staining of Bcl-x$_L$, B cells were fixed and permeabilized by using the CytoFix/Perm Kit (Pharmingen). In short, 5E5 cells were fixed and permeabilized in 0.25 ml of kit buffer overnight at 4°C. Cells were washed twice and stained with 10 μg/ml anti-Bcl-x$_L$ (Santa Cruz Biotechnology) or rabbit IgG (Sigma) and 1% normal mouse serum for 1 h on ice. After washing, cells were then stained with 5 μg/ml phycoerythrin anti-rabbit IgG for 1 h. Cells were fixed in 1% paraformaldehyde and analyzed by flow cytometry by using a FACScan with CELLQUEST software (Becton Dickinson).

Results

LPS and BAFF Preferentially Activate Type 1 or Type 2 NF-κB Pathways, Respectively, Whereas CD40L Strongly Activates both in Primary B Cells. CD40, like BAFF and LPS, can rescue primary B lymphocytes from *in vitro* spontaneous apoptosis and, like LPS, can also induce B cell proliferation and Ig isotype switching. In addition, CD40 has its unique functions in B cell activation such as homotypic aggregation and germinal center formation, which are not shared by either LPS or BAFF. To understand the contribution of both type 1 and type 2 NF-κB pathways in CD40-mediated common (shared by BAFF and LPS) and unique events in B cell activation, we first compared the capacity of CD40, BAFF receptor (BAFF-R), and Toll-like receptor (TLR) 4 (receptor for LPS) to induce these two pathways in the context of primary murine splenic B cells.

First, B cells harvested from WT mice were cultured for 24 h in medium alone or in the presence of 15 μg/ml of an agonistic anti-CD40 mAb, 100 ng/ml hBAFF, or 5 μg/ml LPS (these concentrations were used for all subsequent stimulations unless otherwise stated). Whole-cell lysate was collected, and the processing of p100 to p52 was assessed by Western blot analysis as an indication of the activation of the type 2 pathway. Anti-CD40 and hBAFF treatment, but not LPS treatment, of B cells resulted in the processing of p100 to p52 (Fig. 1*A*). To better understand the kinetics of type 1 and type 2 NF-κB activation by these stimuli, WT B cells were cultured in the presence of anti-CD40 mAb, hBAFF, or LPS for 4, 12, and 24 h. Nuclei were harvested and lysed for detection of p65 (a type 1 indicator) and p52 (from type 2 activation) nuclear translocation by Western blot. Results showed that only CD40 ligation led to sustained nuclear translocation of both p65 and p52, providing a possible explanation for the unique set of biological activities associated with CD40 in comparison with BAFF-R and TLR4 (Fig. 1*B*).

CD40-Stimulated B Cells Retain Significant Survival After Either Single or Compound Loss of Rel Family Members. B cells from WT, p50$^{-/-}$, c-Rel$^{-/-}$, and p52$^{-/-}$ mice were stimulated in medium alone or with anti-CD40 mAb, hBAFF, or LPS for 48 h. Ligand-induced survival is reported as the percentage of specific viability (see Fig. 2) to adjust for differences across the various genotypes for unstimulated cells to remain viable. Whereas CD40-ligated B cells showed a minor reduction in cell survival in each Rel knockout background, hBAFF- and LPS-treated B cells displayed significant reduction in viability in this context. Specifically, the loss of p50 or c-Rel, but not p52, greatly reduced LPS-mediated survival, whereas the loss of p50 or p52, but not c-Rel, greatly reduced the rescue signal provided by hBAFF (Fig. 2*A Upper*). Previous work has shown that compound Rel deficiency, specifically dual loss of p50 and c-Rel, completely abrogates LPS-mediated survival of primary B cells (21). To examine whether the same were true for CD40- and hBAFF-mediated survival, WT, p50$^{-/-}$c-Rel$^{-/-}$, and p50$^{-/-}$c-Rel$^{-/-}$ p65$^{-/+}$ B cells were stimulated as above. As expected, LPS was

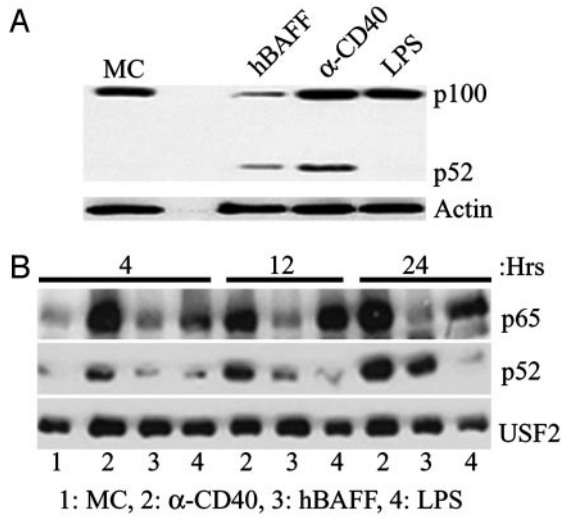

Fig. 1. CD40 ligation, but not hBAFF or LPS treatment, of primary B cells activates both type 1 and type 2 NF-κB activity. (*A*) p100 processing to p52. WT B cells were cultured in medium alone (MC) or in the presence of hBAFF, anti-CD40 mAb, or LPS for 24 h. Whole-cell lysates were Western-blotted for p52 and actin (loading control). (*B*) p65 and p52 nuclear translocation. WT B cells were cultured with hBAFF, anti-CD40 mAb, or LPS for the indicated times. Nuclear extracts were Western-blotted for p65, p52, and upstream stimulatory factor 2 (USF2; loading control). Data are representative of three experiments.

incapable of providing a rescue signal in the compound Rel-deficient backgrounds. CD40 and BAFF-R ligation, on the other hand, retained approximately half of their WT survival signal in these same backgrounds (Fig. 2*A Lower*).

To better understand the capacity of CD40 ligation to elicit significant survival in a compound Rel-deficient background, we compared the ability of anti-CD40 mAb, hBAFF, and LPS treatment to regulate the induction of Bcl-x_L in WT and $p50^{-/-}$c-$Rel^{-/-}p65^{-/+}$ B cells. First, RNA and whole-cell lysate isolated from WT B cells stimulated with anti-CD40 mAb, hBAFF, or LPS was used to analyze Bcl-x_L mRNA and protein by Northern and Western blot, respectively. As shown in Fig. 2*B Left*, anti-CD40 and LPS treatment, but not hBAFF treatment, of B cells led to significant induction of Bcl-x_L mRNA. Interestingly, anti-CD40 treatment was able to maintain Bcl-x_L induction much longer than LPS. In accordance with the Northern blot result, Western blot analysis demonstrated the ability of anti-CD40 and LPS treatment, but not hBAFF treatment, of B cells to induce significant Bcl-x_L protein (Fig. 2*B Right*). In addition, CD40 ligation led to higher levels of Bcl-x_L protein than did LPS treatment, likely because of sustained Bcl-x_L mRNA levels mediated by CD40. Next, WT and $p50^{-/-}$c-$Rel^{-/-}p65^{-/+}$ B cells were stimulated with anti-CD40 mAb or LPS for 24 h. Intracellular staining of Bcl-x_L protein showed that anti-CD40 and LPS treatment of WT B cells led to a significant increase in Bcl-x_L protein in agreement with Western blot results (Fig. 2*C Left*). Simultaneous loss of p50, c-Rel, and one allele of p65, however, completely blocked LPS induction of Bcl-x_L protein whereas CD40-mediated induction, although significantly diminished, was clearly measurable, thus providing some explanation for the capacity of CD40 to elicit significant rescue from apoptosis in the context of compound Rel deficiency.

Type 1 NF-κB Activity Mediated CD40-Induced Proliferation of Primary B Cells. Previous studies have shown type 1 NF-κB activity to be crucial for LPS- and anti-IgM-induced cell cycle entry of primary murine B cells (14, 22). However, the ability of CD40 to activate both type 1 and type 2 NF-κB activity, unlike LPS or anti-IgM, may render CD40-mediated cell cycle entry less sensitive (as seen in CD40-mediated survival) to deficiency in type 1 Rel family members. To examine this possibility, RNA from WT B cells cultured in the presence of anti-CD40 mAb, hBAFF, or LPS was isolated for Northern blot analysis of the G_1–S transition genes *c-Myc*, *CDK4*, and *cyclin D2*. As expected, anti-CD40 and LPS treatment, but not hBAFF treatment, led to marked induc-

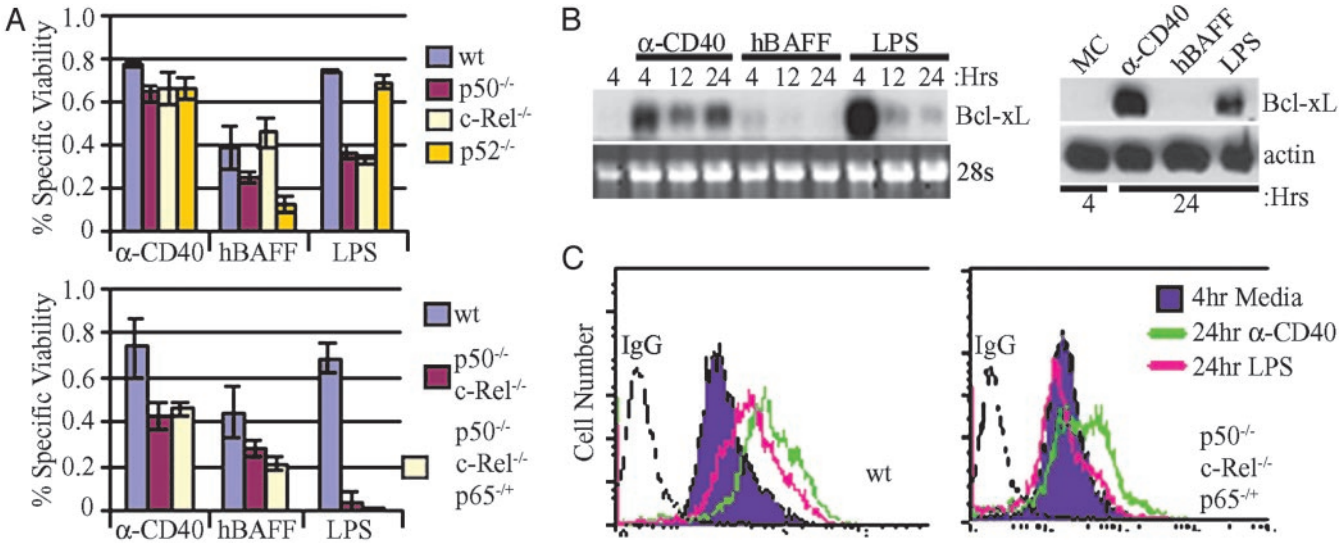

Fig. 2. CD40 ligation, but not hBAFF or LPS treatment, of primary B cells maintains significant signal-induced survival in the context of both single and compound Rel family member deficiency. (*A*) Cell survival. B cells from WT, $p50^{-/-}$, c-$Rel^{-/-}$, $p52^{-/-}$, $p50^{-/-}$c-$Rel^{-/-}$, and $p50^{-/-}$c-$Rel^{-/-}p65^{-/+}$ mice were cultured in triplicate for 48 h with anti-CD40 mAb, hBAFF, or LPS. Propidium iodide-annexin double-negative cells were used to determine the percentage of specific viability [(ligand-mediated viability − medium-mediated)/(100 − medium-mediated)]. Data represent the mean ± SD of three experiments. (*B*) Bcl-x_L induction. RNA and whole-cell extract was prepared from WT B cells cultured with anti-CD40 mAb, hBAFF, or LPS for the indicated times. Bcl-x_L mRNA and protein levels were analyzed by Northern and Western blot, respectively. Actin and 28s RNA levels served as loading controls. (*C*) WT and $p50^{-/-}$c-$Rel^{-/-}p65^{-/+}$ B cells were cultured with medium alone (4 h) and anti-CD40 mAb or LPS (24 h). Cells were permeabilized and stained with anti-Bcl-x_L and rabbit IgG as a control. Intracellular staining was executed twice with similar results.

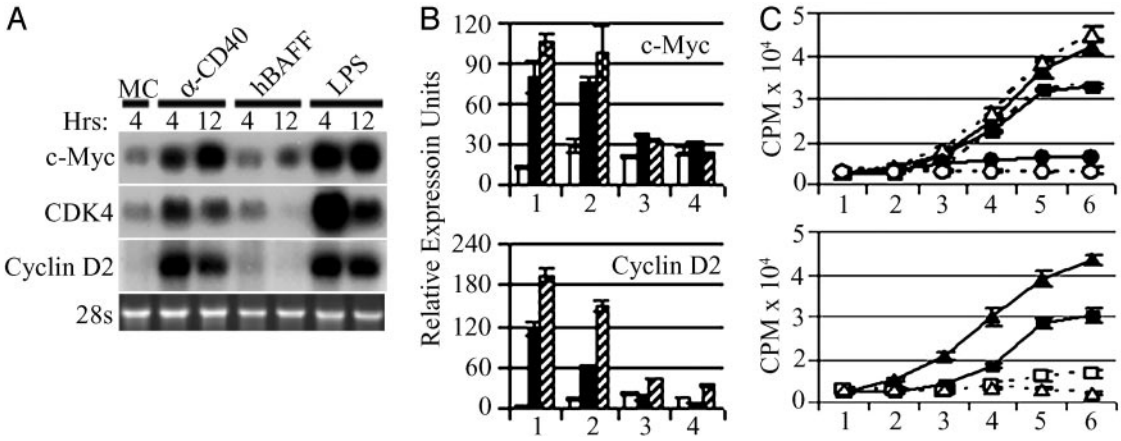

Fig. 3. Loss of type 1, but not type 2, NF-κB activity blocks CD40-mediated proliferation of primary B cells. (*A*) Induction of G_1–S cell cycle transition genes. B cells from WT mice were stimulated with anti-CD40 mAb, hBAFF, or LPS. RNA was collected for analysis of *c-Myc*, *CDK4*, and *cyclin D2* by Northern blot. (*B*) Contribution of type 1 NF-κB to *c-Myc* and *cyclin D2* induction. WT (lane 1), $p50^{-/-}$ (lane 2), $c\text{-}Rel^{-/-}$ (lane 3), and $p50^{-/-}c\text{-}Rel^{-/-}$ (lane 4) B cells were stimulated with medium (open bars), anti-CD40 (filled bars), and LPS (hatched bars) for 12 h. RNA was isolated for Q-PCR analysis of the indicated genes. (*C*) DNA synthesis. WT (solid lines in *Upper* and *Lower*), $p52^{-/-}$ (broken lines in *Upper*), and $p50^{-/-}c\text{-}Rel^{-/-}$ (broken lines in *Lower*) B cells were stimulated in triplicate with a 3-fold dilution series of anti-CD40 mAb (squares), hBAFF (circles), or LPS (triangles) for 48 h. The high dose of each ligand, labeled 6, was 45, 0.9, and 30 μg/ml for anti-CD40 mAb, hBAFF, and LPS, respectively. DNA synthesis was measured by addition of [^{3}H]thymidine at 20 h poststimulation. Data represent the mean ± SD of two experiments.

tion of these genes (Fig. 3*A*). Next, RNA from WT, $p50^{-/-}$, $c\text{-}Rel^{-/-}$, and $p50^{-/-}c\text{-}Rel^{-/-}$ B cells cultured with anti-CD40 mAb or LPS was isolated for cDNA synthesis, and Q-PCR was used for detection of *c-Myc* and *cyclin D2* levels. As shown in Fig. 3*B*, loss of c-Rel was sufficient to block CD40-mediated induction of both *c-Myc* and *cyclin D2*. To further verify that type 1 NF-κB activity controls CD40-mediated cell cycle entry of primary B cells, WT, $p52^{-/-}$, and $p50^{-/-}c\text{-}Rel^{-/-}$ B cells were stimulated for 48 h with titrations of anti-CD40 mAb or LPS. Cell cycle entry was measured by means of addition of [^{3}H]thymidine 20 h poststimulation. Although loss of p52 did not affect either CD40- or LPS-mediated DNA synthesis (Fig. 3*C Upper*), CD40-mediated cell cycle entry was blocked in the context of compound type 1 Rel deficiency, as published for LPS and anti-IgM (Fig. 3*C Lower*).

CD40-Mediated Homotypic Aggregation of B Cells Requires Type 1 and Type 2 NF-κB Pathways. CD40 ligation on primary B cells is known to induce striking and highly stable homotypic aggregates by 24 h of culture (23, 24). To examine the contribution of both type 1 and type 2 NF-κB pathways in CD40-mediated homotypic aggregation, WT, $p50^{-/-}$, $c\text{-}Rel^{-/-}$, and $p52^{-/-}$ B cells were stimulated with anti-CD40 mAb, hBAFF, or LPS for 24 h. As shown in Fig. 4, anti-CD40 treatment, but not hBAFF or LPS treatment, of WT cells induced B cell aggregation. In addition, CD40-mediated homotypic aggregation was found to require the presence of both c-Rel and p52. Some have speculated that an unidentified B cell chemokine may also be involved in CD40-mediated B cell homotypic aggregation. To explore this possibility, conditioned medium from WT B cells stimulated with anti-CD40 mAb for 24 h was supplied with fresh Ab and used to stimulate $c\text{-}Rel^{-/-}$ and $p52^{-/-}$ B cells. No rescue of homotypic aggregation was observed (data not shown).

Complete CD40-Mediated Gene Program Requires Activation of Type 1 and Type 2 NF-κB Pathways. To further address the significance of CD40-mediated activation of both type 1 and type 2 NF-κB pathways in primary B cells, we sought to compare CD40-mediated gene activation with that elicited by LPS or hBAFF. Through analysis of a gene panel including targets induced by both anti-CD40 and LPS treatment (but not hBAFF treatment) of B cells and genes common to anti-CD40 and hBAFF treatment (but not LPS treatment) of B cells, we could test the prediction that the former category would be sensitive to the loss of type 1 NF-κB activity and that the latter would be sensitive to the loss of type 2 NF-κB activity. First, Northern blot analyses of RNA purified from WT B cells cultured with anti-CD40 mAb, hBAFF, or LPS revealed gene(s) whose activation was common to CD40 and TLR4 ligation [*AID* and *UNG*, both crucial to Ig isotype switching and affinity maturation (25–28)], specific to CD40 ligation [*Entactin*, important in lymphocyte adhesion/migration (29, 30)], and common to CD40 and BAFF-R ligation [*LEF-1*, involved in pro/pre-B cell development and proliferation (31)] (Fig. 5*A*). To determine the contribution of type 1 and type 2 NF-κB pathway activation to induction of these genes, RNA was isolated from WT, $p50^{-/-}$, $c\text{-}Rel^{-/-}$, and $p52^{-/-}$ B cells stimulated with anti-CD40 mAb and analyzed by Northern blot (Fig. 5*B*). As predicted, genes activated by CD40 and TLR4 ligation and not BAFF-R, namely *AID* and *UNG*, required type 1 and not type 2 NF-κB activity. Specifically, loss of p50 or c-Rel prevented CD40-mediated induction of *AID*, whereas loss of

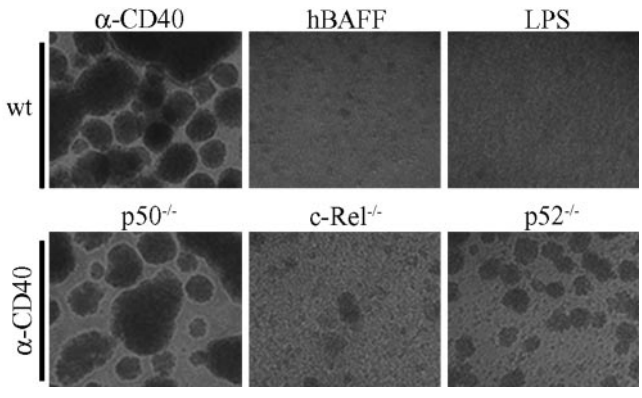

Fig. 4. CD40-mediated homotypic aggregation of primary B cells requires both type 1 and type 2 NF-κB activity. Homotypic aggregation is shown. 5E6 B cells from WT, $p50^{-/-}$, $c\text{-}Rel^{-/-}$, and $p52^{-/-}$ mice were stimulated in a 24-well format for 24 h with anti-CD40 mAb, hBAFF, or LPS. Pictures are of representative fields at ×4 magnification.

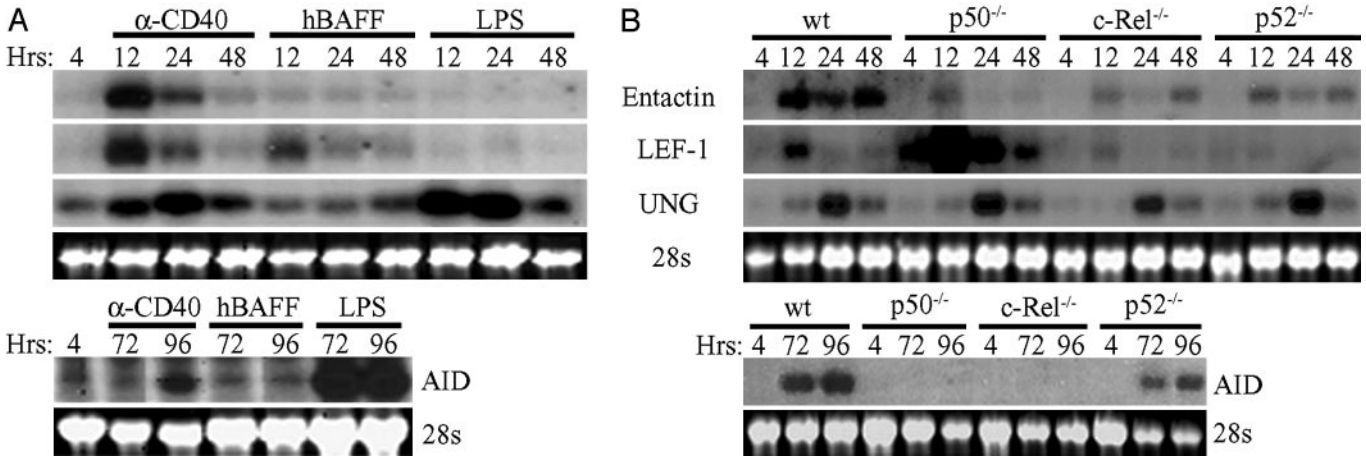

Fig. 5. Type 1 and type 2 NF-κB pathways mediate the CD40-induced gene program in primary B cells. (*A*) Gene induction program. WT B cells were cultured in medium alone (4 h) and in the presence of anti-CD40 mAb, hBAFF, or LPS over an extended time course. RNA was purified, and the indicated genes were analyzed by Northern blot. (*B*) Type 1 and type 2 NF-κB contribution to CD40-induced genes. WT, $p50^{-/-}$, $c\text{-}Rel^{-/-}$, and $p52^{-/-}$ B cells were cultured in medium alone (4 h) and in the presence of anti-CD40 mAb over an extended time course. Purified RNA was isolated, and the indicated genes were analyzed by Northern blot. Representative gene induction profiles are shown.

c-Rel, but not p50 or p52, delayed and reduced CD40-mediated induction of *UNG*. Furthermore, CD40-mediated induction of *LEF-1* required p52, whereas loss of p50 actually enhanced induced mRNA levels. Interestingly, the CD40-specific target, *Entactin*, was found to require both type 1 and type 2 NF-κB pathways because loss of p50, c-Rel, or p52 markedly reduced induced message levels.

Discussion

Primary B cells can be activated by means of a diverse set of surface receptors, including mediators of both innate and adaptive immunity. Upon ligation, such receptors, including CD40, BCR, TLR4, and TLR9, activate similar biochemical pathways, making it difficult to explain the mechanism of divergence in their respective gene expression profiles and induced cellular responses. However, studies on the mechanism of combinatorial control of gene regulation have revealed that variation in biochemical pathway activation results in marked differences in gene activation potential.

Previous work in our laboratory used high-density oligonucleotide arrays to compare the gene expression profiles of CD40L and LPS treatment of primary murine B cells (17). Although these stimuli share the ability to activate the c-Jun N-terminal kinase/stress-activated protein kinase, p38, phosphatidylinositol 3-kinase, and extracellular signal-regulated kinase/mitogen-activated protein kinase pathways, in addition to type 1 NF-κB activity, we identified gene activation unique to each ligand. Subsequent work revealed the capacity of LPS to specifically activate the transcription factor IFN regulatory factor 3 (32), which, together with NF-κB, mediated the induction of a large set of the LPS-specific genes identified in our previous microarray study (20). In this work, we explored the contribution of type 1 and type 2 NF-κB pathways to B cell activation, and we provide evidence of type 2 NF-κB activity as a possible explanation for unique CD40-mediated gene activation and cellular responses.

Primary B cells rapidly undergo spontaneous apoptosis in culture even in the presence of serum. Although treatment with CD40L, BAFF, and LPS can all rescue B cells from apoptosis, we found that they had differential requirements for individual NF-κB subunits (see Fig. 2*A Upper*). In addition, whereas compound deficiency in type 1 Rel members completely blocked LPS-mediated survival, CD40 ligation retained a significant survival signal in this context (see Fig. 2*A Lower*). Intracellular staining of $Bcl\text{-}x_L$ verified the capacity of CD40 ligation to induce a survival factor in $p50^{-/-}c\text{-}Rel^{-/-}p65^{-/+}$ B cells (see Fig. 2*C*). Although we cannot exclude the contribution of other survival pathways, the remaining NF-κB activity, especially the ability of CD40 ligation to activate both type 1 and type 2 NF-κB pathways, is most likely involved in CD40-mediated survival of these cells, because the total NF-κB inhibitors, Gliotoxin (20S and 26S proteasome inhibitor) and SN50 (a cell-permeable peptide that binds the Rel nuclear localization sequence), completely blocked CD40-mediated survival (data not shown). In support of this, we found that loss of p52 had an even greater affect than loss of p50 (33) on the ability of hBAFF to provide a rescue signal to B cells, implying that p52 can replace a significant portion of CD40-mediated survival lost in the absence of multiple type 1 Rel family members.

In contrast to cell survival, CD40-mediated induction of genes involved in cell proliferation (*c-Myc*, *CDK4*, or *cyclin D2*) and Ig isotype switching (*AID* and *UNG*) seems to depend mostly on type 1 but not type 2 NF-κB activity. This is consistent with the observation that CD40L and LPS but not BAFF can strongly promote resting B lymphocytes for cell cycle entry and proliferation. Our gene expression studies are also consistent with previous functional studies indicating that deficiency in either p50 or c-Rel prevents Ig isotype switching elicited by multiple stimuli (13, 34).

In addition to common functions such as proliferation and survival, CD40 ligation of primary B cells induces cellular responses not shared by either LPS or hBAFF. Our analysis of type 1 and type 2 NF-κB contribution supports a model by which CD40L, LPS, and hBAFF common outcomes result from the shared capacity to activate either type 1 or type 2 NF-κB, whereas the unique functions of CD40 result from the capacity to activate both pathways (Fig. 6). Indeed, examination of CD40-mediated homotypic aggregation revealed that both pathways are required, because loss of c-Rel or p52 greatly reduced the formation of stable aggregates (see Fig. 4). In addition, Northern blot analysis of type 1 and type 2 NF-κB contribution to CD40-mediated induction of *Entactin* and *LEF-1* (two genes induced by CD40L but not LPS by microarray analysis) revealed that *LEF-1* required the presence of p52 whereas *Entactin* required both type 1 and type 2 NF-κB activity. Further studies are required to examine the mechanism of cooperative type 1

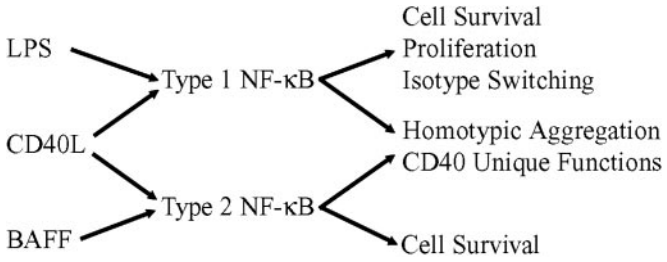

Fig. 6. A model for the contribution of type 1 and type 2 NF-κB in LPS-, CD40L-, and BAFF-induced B cell activation. CD40L activation of primary B cells shares common cellular outcomes with both LPS- and BAFF-mediated activation. In each case, these shared functions result from the shared capacity of these ligands to activate either type 1 or type 2 NF-κB activity. In addition, CD40L's ability to activate both type 1 and type 2 NF-κB activity results in unique, CD40-specific B cell responses.

and type 2 NF-κB activity to unique CD40-mediated B cell responses. Although CD40 ligation of B cells leads to the immediate and sustained activation of the type 1 pathway, strong activation of the type 2 pathway, for poorly understood reasons, requires substantial time. It is still not clear whether unique CD40-mediated B cell responses require specific periods of type 1 and type 2 NF-κB synergy. To this point, it is worth noting that combinatorial treatment of WT B cells with LPS and hBAFF, which results in the activation of both type 1 and type 2 NF-κB (data not shown), did not induce homotypic aggregates after 24 h of stimulation (data not shown). Interestingly, this combinatorial treatment did result in the formation of spherical clusters after 72 h of stimulation, although not to the extent of CD40 ligation (B.Z. and G.C., unpublished observations).

Thus, our studies reveal that the CD40-mediated gene expression and biological program require dual activation of the type 1 and type 2 NF-κB pathways. Of course, CD40-mediated responses in B cells require the activation of additional biochemical pathways [e.g., p38 and extracellular signal-regulated kinase kinase pathways (17)]. Continued investigation is required to understand how these signaling circuits cooperate in CD40 signal transduction and cellular responses. Our results are a reminder that the mechanisms by which cells execute diverse functions rely on the ability of different combinations of distinct but mostly shared stimuli to elicit divergent cellular responses. This phenomenon is no better exemplified than in CD40-mediated, T cell-dependent immune responses in which activated B cells, over many days, must integrate dozens of changing signals to achieve complex ends, including memory and plasma B cell differentiation.

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