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# I $\kappa$ B $\epsilon$ Is a Key Regulator of B Cell Expansion by Providing Negative Feedback on cRel and RelA in a Stimulus-Specific Manner

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The transcription factor NF- $\kappa$ B is a regulator of inflammatory and adaptive immune responses, yet only I $\kappa$ B $\alpha$  was shown to limit NF- $\kappa$ B activation and inflammatory responses. We investigated another negative feedback regulator, I $\kappa$ B $\epsilon$ , in the regulation of B cell proliferation and survival. Loss of I $\kappa$ B $\epsilon$  resulted in increased B cell proliferation and survival in response to both antigenic and innate stimulation. NF- $\kappa$ B activity was elevated during late-phase activation, but the dimer composition was stimulus specific. In response to IgM, cRel dimers were elevated in I $\kappa$ B $\epsilon$ -deficient cells, yet in response to LPS, RelA dimers also were elevated. The corresponding dimer-specific sequences were found in the promoters of hyperactivated genes. Using a mathematical model of the NF- $\kappa$ B–signaling system in B cells, we demonstrated that kinetic considerations of I $\kappa$ B kinase–signaling input and I $\kappa$ B $\epsilon$ 's interactions with RelA- and cRel-specific dimers could account for this stimulus specificity. cRel is known to be the key regulator of B cell expansion. We found that the RelA-specific phenotype in LPS-stimulated cells was physiologically relevant: unbiased transcriptome profiling revealed that the inflammatory cytokine IL-6 was hyperactivated in I $\kappa$ B $\epsilon$ <sup>-/-</sup> B cells. When IL-6R was blocked, LPS-responsive I $\kappa$ B $\epsilon$ <sup>-/-</sup> B cell proliferation was reduced to near wild-type levels. Our results provide novel evidence for a critical role for immune-response functions of I $\kappa$ B $\epsilon$  in B cells; it regulates proliferative capacity via at least two mechanisms involving cRel- and RelA-containing NF- $\kappa$ B dimers. This study illustrates the importance of kinetic considerations in understanding the functional specificity of negative-feedback regulators. *The Journal of Immunology*, 2014, 192: 3121–3132.

The NF- $\kappa$ B family of transcription factors controls expression of an extensive array of genes responsible for cell survival, proliferation, inflammation, and immune regulation. This transcription factor family consists of a variety of dimers formed by combinations of five Rel homology-containing proteins: RelA, RelB, cRel, p50, and p52. The activities of these dimers are regulated by members of the classical I $\kappa$ B protein family: I $\kappa$ B $\alpha$ , I $\kappa$ B $\beta$ , and I $\kappa$ B $\epsilon$ . I $\kappa$ B proteins limit NF- $\kappa$ B activity in the cellular basal state but allow for NF- $\kappa$ B activation when inflammatory stimuli result in their N-terminal–specific serine phos-

phorylation by the NEMO-containing I $\kappa$ B kinase (IKK) complex, specific lysine ubiquitination, and subsequent proteasome-dependent degradation (1). However, NF- $\kappa$ B activity is dynamic and transient. Both I $\kappa$ B $\alpha$  and I $\kappa$ B $\epsilon$  are transcriptionally induced by NF- $\kappa$ B, yet only I $\kappa$ B $\alpha$  provides critical negative-feedback functions (2, 3).

Although these insights have largely been derived from convenient cell line systems, such as HeLa cells and mouse embryonic fibroblasts (MEFs), NF- $\kappa$ B's major physiological functions are in lymphocytes, where it has a key role in regulating proliferation and survival during the adaptive-immune response (4–14). Although RelA:p50 is the predominant dimer in HeLa cells and MEFs, in B cells, upon activation via antigenic stimulation through the BCR by anti-IgM or pathogenic stimulation through the TLR by LPS, there is a significant increase in nuclear DNA binding activity of both RelA:p50 and cRel:p50 dimers (9, 10, 12, 15). Interestingly, the majority of the evidence supports a critical role for cRel and p50 (16–19), but not RelA (20), in controlling B cell proliferation.

Although it is understood that the cRel:p50 dimer plays an essential role in B cell proliferation and survival, little is known about the mechanisms responsible for controlling its activity. In fibroblasts, I $\kappa$ B $\alpha$  is known to be the primary regulator of the ubiquitous RelA:p50 dimer; I $\kappa$ B $\alpha$ -deficient fibroblasts show elevated basal levels, reduced activation, and prolonged duration of RelA:p50 activity in response to stimulation by the proinflammatory cytokine TNF- $\alpha$  (21). I $\kappa$ B $\epsilon$  provides a secondary role, partially compensating for I $\kappa$ B $\alpha$  deficiency, but I $\kappa$ B $\epsilon$  deficiency alone shows no discernible phenotype. Biochemical characterization suggests that I $\kappa$ B $\alpha$  preferentially binds RelA:p50 dimers, whereas I $\kappa$ B $\epsilon$  associates with RelA- and cRel-containing dimers (22–24). These differences suggest that I $\kappa$ B $\alpha$  and I $\kappa$ B $\epsilon$  may have distinct physiological roles in controlling NF- $\kappa$ B dimers. I $\kappa$ B $\epsilon$  was reported to be a nonredundant regulator

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Abbreviations used in this article: 7-AAD, 7-aminoactinomycin D; FO, follicular; FPKM, fragments per kb of exon per million fragments mapped; IKK, I $\kappa$ B kinase; MEF, mouse embryonic fibroblast; MZ, marginal zone; pF, progressor fraction; Tdie0, time to death; Tdiv0, time to the first division.

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of cRel-dependent expression of BAFF receptor and CD40 (25), but how it controls cRel-containing dimers or what other genes may be regulated remain unclear.

In this study, we investigated the role of I $\kappa$ B $\epsilon$  in controlling NF- $\kappa$ B activity in B lymphocytes.

Our results indicate that the ablation of I $\kappa$ B $\epsilon$  allows for increased proliferation and survival in B cells stimulated with either IgM or LPS. In fact, we found that I $\kappa$ B $\epsilon$  had a role in limiting cRel- and RelA-containing dimers, albeit in a stimulus-specific manner, as evidenced by both biochemical data and DNA motif signatures in hyperregulated genes. Mathematical modeling was used to show that a consideration of known kinetic differences between these proteins provides a sufficient explanation. Further, we found that I $\kappa$ B $\epsilon$  control of RelA in response to LPS was functionally relevant, because hyperinduction of IL-6 in I $\kappa$ B $\epsilon$ -deficient B cells was shown to mediate hyperexpansion.

## Materials and Methods

### Cell isolation and culture

Spleens were harvested from C57Bl6 wild-type (WT) mice (The Jackson Laboratory, Bar Harbor, ME) and C57Bl6 I $\kappa$ B $\epsilon$ <sup>-/-</sup> mice (2). The collected spleens were homogenized by grinding between frosted glass slides. For B cell isolation, homogenized splenocytes were incubated with anti-CD43 (Ly-48) microbeads for 15 min at room temperature. Following this incubation, cells were washed with HBSS (Life Technologies; cat. no. 14170) containing 1% FCS, 10 mM HEPES (Life Technologies; cat. no. 15630), and 1% FCS (Sigma; cat. no. F2442) and separated over a magnetic column (LS column; Miltenyi Biotec; cat. no. 130-042-401). For B cells, purity was determined by flow cytometry using PE anti-B220 (cat. no. 12-0452-83), FITC anti-CD3 (cat. no. 11-0031-82), allophycocyanin anti-CD4 (cat. no. 17-0041-83), and PerCP anti-CD8 (cat. no. 46-0081-82; all from eBioscience). Purity was consistently between 92 and 95% (data not shown). For experiments in which separation of marginal zone (MZ) B cells and follicular (FO) B cells was performed, anti-CD43-magnetically separated B cells were stained with allophycocyanin anti-CD9 (eBioscience; cat. no. 17-0091-82), and B cell population separation was performed using a FACSAria II cell sorter (BD Biosciences). Complete media consists of RPMI 1640 (cat. no. 11875), 10 mM HEPES (cat. no. 15630), 1 mM sodium pyruvate (cat. no. 11360), 1 mM nonessential amino acids (cat. no. 11140), 0.055 mM 2-ME (cat. no. 21985), 100 U Penicillin/Streptomycin (10378016; all from Life Technologies), and 0.3 mg/ml glutamine. B cells were stimulated with either 10  $\mu$ g/ml anti-IgM (Jackson ImmunoResearch; cat. no. 115-006-020) or 10  $\mu$ g/ml LPS (Sigma; cat. no. L2630).

### Flow cytometry analysis of cell proliferation and survival

Purified B cells were stained with 5  $\mu$ M CFSE (Invitrogen; cat. no. C1157) and cultured in complete media with previously mentioned stimuli (see above). B cells were collected at various time points and stained with 7-aminoactinomycin D (7-AAD; Invitrogen; cat. no. A1310). B cells were analyzed for proliferation and survival using a C6 Accuri flow cytometer (BD Biosciences). Gating of live B cells was determined by forward scatter and side scatter properties. Differences in cell proliferation were measured using FlowJo software (TreeStar), as well as FloMax (26), which determines maximum-likelihood cellular parameter sets for fractions of responding cells, times to division, and times to death for generations 0 to 10.

### EMSA and supershifts

Nuclear extracts were generated from B cells using high salt extraction. In brief, purified B cells were incubated with a low-salt buffer (10 mM HEPES (pH 7.9); Life Technologies), 10 mM KCl [Thermo Fisher Scientific; cat. no. P217], 0.1 mM EGTA [Sigma; E-4378], 0.1 mM EDTA [Thermo Fisher Scientific; cat. no. S312], 1 mM DTT [Thermo Fisher Scientific; BP172-5], 1 mM PMSF [Sigma; cat. no. P7626], 5  $\mu$ g/ml apoprotein [Sigma; cat. no. A1153], 5  $\mu$ g/ml leupeptin [Sigma; cat. no. L2884], 1  $\mu$ M pepstatin A [Sigma; cat. no. P5318] for 10 min on ice. Following this incubation, cells were disrupted through the addition of Nonidet P-40 (US Biological; cat. no. N3500) to a final concentration of 0.5% and vortexing for 15 s. Nuclei were pelleted away from the cytoplasmic fraction by centrifugation at 15,000 rpm for 1 min, and the cytoplasmic fraction was pipetted into a separate tube. The remaining nuclei were disrupted by a 20-min incubation at 4°C in a high-salt buffer ([20 mM HEPES (pH 7.9); Life Technologies], 400 mM NaCl [Thermo Fisher Scientific; cat. no. S671], 1 mM EGTA [Thermo Fisher Scientific], 1 mM EDTA [Thermo Fisher Scientific], 20%

glycerol [Thermo Fisher Scientific], 1 mM DTT [Thermo Fisher Scientific], and 1 mM PMSF [Sigma]). The nuclear fraction was collected following centrifugation at 15,000 rpm for 5 min. Equal amounts of nuclear extracts (1  $\mu$ g) were preincubated for 20 min on ice in the presence or absence of Abs specific for RelA (cat. no. sc-372), RelB (cat. no. sc-226), or cRel (cat. no. sc-71; all from Santa Cruz Biotechnology) or in the following combinations: RelA/RelB, RelA/cRel, and RelB/cRel. Following the preincubation with Abs, ( $\gamma$ -<sup>32</sup>P)ATP (GE Health)-radiolabeled probe derived from HIV- $\kappa$ B sequence 5'-GCTACAAGGGACTTTCCGC-TGGGGACTTTCAGGGAGG-3' was added and incubated at room temperature for an additional 15 min. The resulting DNA/protein/Ab complexes were resolved by electrophoresis on a 5% nondenaturing polyacrylamide gel and exposed to storage phosphor screen (GE Healthcare) overnight before image development on a Typhoon 9200 Variable Mode Imager (GE Healthcare). Images were analyzed and quantitated using ImageQuant (GE Healthcare).

### Western blot analysis

Whole-cell lysates were prepared using RIPA buffer lysis of B cells. Cytoplasmic extracts and nuclear extracts were prepared as previously described (27, 28). The resulting lysates and extracts were run on either 10% SDS-PAGE gels or 5–14% Criterion Tris-HCl Gel (Bio-Rad). The following Abs were used to identify the protein of interest: p65, I $\kappa$ B $\alpha$ , cRel, and actin (all from Santa Cruz Biotechnology). The resulting proteins were detected using the Bio-Rad ChemiDoc XRS System and SuperSignal West Femto Substrate Maximum Sensitivity Substrate (Thermo Scientific) to detect chemiluminescence released by HRP-labeled secondary Abs.

### Cytokine neutralization and receptor blocking

CFSE-labeled B cells isolated from either WT or I $\kappa$ B $\epsilon$ <sup>-/-</sup> B cells were stimulated with either 10  $\mu$ g/ml IgM or 10  $\mu$ g/ml LPS in the presence of 2  $\mu$ g/ml the following Abs: anti-mouse IL-1 $\alpha$ /IL-1F1 AF-400-NA, anti-mouse IL-1 $\beta$ /IL-1F2 AF-401-NA, anti-mouse IL-6 AF-406-NA, anti-mouse IL-1R AF-480-NA, and anti-mouse IL-6R $\alpha$  AF1830 (all from R&D Systems). Cell proliferation was measured using a C6 Accuri flow cytometer (BD Biosciences). Differences in cell proliferation were measured using FlowJo software (TreeStar).

### ELISAs

Isolated B cells were plated at a concentration of 2 million cells/ml in the presence of 10  $\mu$ g/ml IgM or 10  $\mu$ g/ml LPS. Cells were harvested at 2, 4, 8, 22, 34, and 48 h and spun down, and the supernatant was collected. The resulting supernatant was tested for measured release of IL-1 $\alpha$  (Mouse IL-1 $\alpha$  ELISA MAX Deluxe), IL-1 $\beta$  (Mouse IL-1 $\beta$  ELISA MAX Deluxe), and IL-6 (Mouse IL-6 ELISA MAX Deluxe; all from BioLegend).

### Transcriptome analysis

Total RNA was isolated from IgM- or LPS-stimulated B cells isolated from WT or I $\kappa$ B $\epsilon$ <sup>-/-</sup> B cells over a four-point time course. mRNA was extracted from 2  $\mu$ g total RNA using oligo(dT) magnetic beads and fragmented at high temperature using divalent cations. Next, a cDNA library was generated using Illumina TruSeq kits, and quantitation was performed using the Roche LightCycler 480. Sequencing was performed on Illumina's HiSeq 2000, according to the manufacturer's recommendations, by the Biomedical Genomics Microarray Core facility at the University of California, San Diego. Reads were aligned to the mouse mm10 genome and RefSeq genes (PMID 12045153, PMID 12466850) with Tophat (PMID 19289445). Cufflinks and Cuffdiff were used to ascertain differential expression of genes. Gene differential fragments per kb of exon per 1 million fragments mapped (FPKM)s were obtained from the cuffdiff program in the Tuxedo RNA sequencing analysis suite. In R, we took the log base 2 of the FPKM values and used the WT 0 h to normalize. Induced genes are identified as genes that have a 2-fold increase in expression above basal (0 h) in at least one time point. Hyperinduced is defined as genes in I $\kappa$ b $\epsilon$  knockout B cells that have a >2-fold increase in expression above their WT counterpart at two or more non-0 h time points. Using R's gplots package, heat maps were created for fold change for all genes that made the above induction cut-offs. Additionally, heat maps for latent genes, induced no more than 0.3 log base 2 above their corresponding WT counterpart, were created. For both heat maps, the lists of genes mapped were recorded. The above process was done for both IgM- and LPS-stimulated conditions. Determination of NF- $\kappa$ B motifs containing genes was performed using the NF- $\kappa$ B position weight matrix previously identified by Bulyk and colleagues (29) and converted into formats for HOMER motif discovery software (30). The promoter regions of genes found to be either induced or hyperinduced were searched for the occurrence of these

motifs. The percentage of genes from the identified gene lists that contained each motif versus the percentage of promoters containing the NF- $\kappa$ B motifs from genes identified as induced were graphed. Significance was determined using R's stats package to perform a Pearson  $\chi^2$  test using the background counts as the expected values versus the percentage of induced or hyper-induced genes containing the NF- $\kappa$ B motifs.

#### Computational modeling of NF- $\kappa$ B dimer activity

The computational model appends the previously published I $\kappa$ B models with the reactions that govern the generation of RelA- and cRel-containing NF- $\kappa$ B dimers (Supplemental Fig. 3). The model contains 36 species and 146 reactions (Supplemental Model Equations) governed by 74 parameters (Supplemental Table I). Ordinary differential equations were solved numerically using MATLAB version R2013a (The MathWorks) with sub-routine ode15s, a variable order, multistep solver. Prior to stimulation, the system was allowed to equilibrate from starting conditions to a steady-state, defined as showing no concentration changes  $> 1\%$  over a period of 4000 min. Stimulus-induced perturbation from the steady-state was accomplished by direct modulation of IKK activity via a numerical input curve representing IgM or LPS stimulation (adapted from Ref. 31). MATLAB model codes are available upon request.

#### Animal use

The animal protocols for this study were approved by the University of California, San Diego Animal Care and Use Committee.

## Results

### *I $\kappa$ B $\epsilon$ deficiency in B cell subsets results in increased stimulus-responsive proliferation and survival*

Because NF- $\kappa$ B controls B cell expansion, we sought to determine I $\kappa$ B regulators that limit NF- $\kappa$ B activity in B cells and, thus, B cell proliferation. Examining whole-cell extracts, we found that, although I $\kappa$ B $\alpha$  protein levels rapidly decrease upon B cell stimulation with IgM or LPS, I $\kappa$ B $\epsilon$  protein levels decrease only slightly after stimulation and increase at late time points (Supplemental Fig. 1A), suggesting a role in the postinduction attenuation of NF- $\kappa$ B.

Using B cells magnetically purified from mixed splenocytes collected from WT or I $\kappa$ B $\epsilon^{-/-}$  mice, we examined B cell expansion following ex vivo stimulation with 10  $\mu$ g/ml IgM or 10  $\mu$ g/ml LPS using CFSE dye dilution. We found that B cells lacking I $\kappa$ B $\epsilon$  displayed increased expansion with either stimulus compared with WT B cells (Fig. 1A). Several repeats of the CFSE experiments yielded highly reproducible results (Fig. 1B). To determine whether these differences were the result of a proliferation or survival defect, we used the computational phenotyping tool FloMax (26), which parameterizes a modified cyton model (fcyton) to CFSE time courses and yields maximum likelihood nonredundant cellular parameters, such as the percentage of cells entering the proliferative program [progressor fraction (pF)], the time to the first division (Tdiv0), and the time to death (Tdie0) of cells not entering the proliferative program (Fig. 1C). Using FloMax, the CFSE data indicate that I $\kappa$ B $\epsilon^{-/-}$  B cells are more likely to respond to the stimulus (pF<sub>0</sub>) than are WT cells under the same conditions (Fig. 1D). In response to IgM, 51.7% of I $\kappa$ B $\epsilon^{-/-}$  B cells entered division compared with only 24% of WT B cells. Following LPS stimulation, 64% of the I $\kappa$ B $\epsilon^{-/-}$  B cells entered division compared with 52% of their WT counterparts. Because only nonresponding cells are susceptible to death (32–34), and the Tdie0 and Tdiv0 parameters showed little change in the knockout, this suggested that the death rates in I $\kappa$ B $\epsilon^{-/-}$  cells would be lower. Testing this prediction with 7-AAD staining of responding cells at 24 h, we indeed found lower percentages of dying cells in I $\kappa$ B $\epsilon^{-/-}$  cells than in WT populations (Fig. 1E, 1F; 33.3% versus 54.8% in response to IgM, and 21.9% versus 37.7% in response to LPS).

Examining the B cell subsets in the spleen, we observed a higher percentage of MZ B cells compared with FO B cells in I $\kappa$ B $\epsilon^{-/-}$

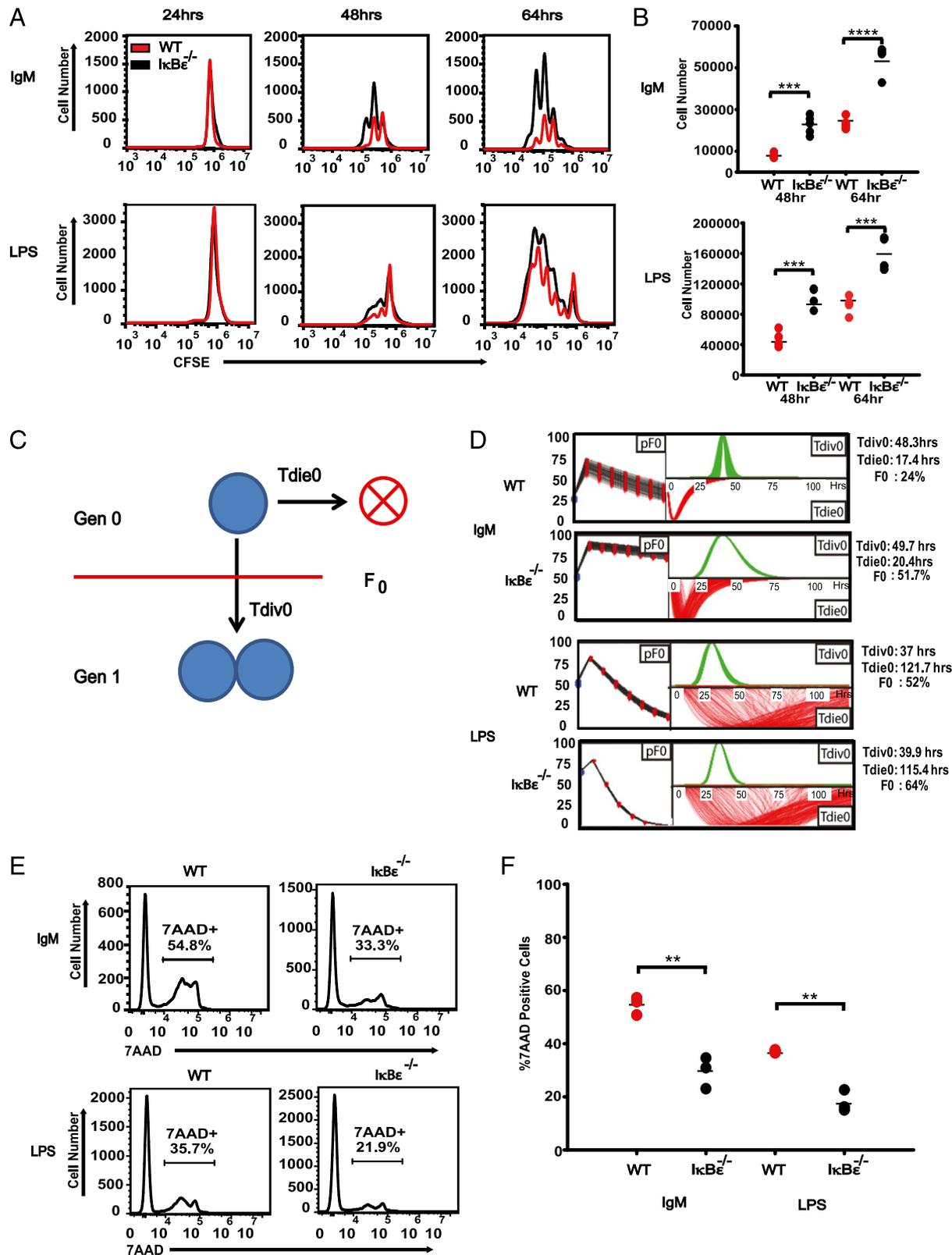
mice compared with WT controls (Fig. 2A). Because MZ B cell maturation is more sensitive to NF- $\kappa$ B activity than is FO B cell maturation, this observation is consistent with elevated NF- $\kappa$ B activity following I $\kappa$ B $\epsilon$  ablation. It also prompted us to question whether the skewed distributions were responsible for the ex vivo expansion phenotype. To address this concern, we purified WT and I $\kappa$ B $\epsilon^{-/-}$  MZ and FO B cells away from each other, using allophycocyanin anti-CD9 staining in conjunction with FACS cell sorting (35). Purity was consistently between 90 and 100% (Fig. 2B). Purified FO B cells, whose physiological role is to respond to Ags, showed increased B cell expansion in response to IgM stimulation when derived from I $\kappa$ B $\epsilon^{-/-}$  mice. Similarly, MZ B cells, whose physiological role is to monitor for circulating endotoxin, showed increased proliferation when derived from I $\kappa$ B $\epsilon^{-/-}$  mice (compared with WT) and stimulated with LPS (Fig. 2C, 2D). These results indicate that the difference in proliferation and survival is a cell-intrinsic B cell phenotype rather than a result of developmental changes that result in differences in the distributions of B cell subpopulations.

### *I $\kappa$ B $\epsilon$ provides negative feedback on both RelA- and cRel-containing dimers, albeit stimulus specifically*

Given the B cell-proliferation phenotype of I $\kappa$ B $\epsilon$  deficiency, we sought to determine how the loss of I $\kappa$ B $\epsilon$  affects NF- $\kappa$ B dimer activation. In previous studies, I $\kappa$ B $\epsilon$  was shown to bind preferentially to cRel (22, 24), suggesting that I $\kappa$ B $\epsilon$  deficiency may preferentially affect cRel-containing dimer activity. We prepared nuclear extracts from WT and I $\kappa$ B $\epsilon^{-/-}$  B cells stimulated with IgM or LPS, as previously described, and used them for EMSAs with a  $\kappa$ B site-containing probe. NF- $\kappa$ B heterodimers (all containing p50, but containing either RelA, cRel, or RelB; see below) were elevated in I $\kappa$ B $\epsilon^{-/-}$  B cells at later time points (18 and 24 h) compared with WT B cells (Fig. 3A), consistent with the high level of induction of this inhibitor seen in immunoblots of WT cells (Supplemental Fig. 1A). Supershift analysis of these B cells' nuclear extracts using specific Abs for the three activation domain-containing Rel proteins, RelA, cRel, and RelB, was used to quantify both the supershifted complex observed with one Ab, as well as the remaining nonsupershifted complex when two Abs were used to ablate the activities of their cognate Rel proteins. We found greatly increased levels of cRel:p50 dimer activity in I $\kappa$ B $\epsilon^{-/-}$  B cell extracts stimulated with either IgM or LPS (Fig. 3B, 3C). Interestingly, examining RelA activity, we found that RelA nuclear activity increased significantly in LPS-stimulated I $\kappa$ B $\epsilon^{-/-}$  B cells compared with WT B cells but not when stimulated with IgM (Fig. 3B, 3C). No difference was seen between the basal levels of RelA and cRel (Supplemental Fig. 1B), suggesting that this effect is induced during stimulation of the B cells. We used immunoblots of nuclear extracts to examine the results further. Following three biological repeats, we found statistically significant differences only for the hyperactivation of cRel in response to LPS, whereas other conditions showed the same trend as the EMSA results but did not achieve statistical significance (Fig. 3D). Together, these biochemical data suggest that I $\kappa$ B $\epsilon$  provides a key function in limiting cRel-containing dimer activity via a negative-feedback loop, whereas it is critical for limiting RelA activity in response to some stimuli but not others.

### *A mathematical model of RelA and cRel dynamics suggests a kinetic basis for I $\kappa$ B $\epsilon$ 's stimulus-specific functions*

The observation that the signaling phenotypes are stimulus specific may suggest that there are underlying stimulus-specific biochemical mechanisms, such as a costimulatory signaling pathway, that are activated by one stimulus but not another. An alternative, more parsimonious explanation is that differential-signaling



**FIGURE 1.** I $\kappa$ B $\epsilon$ <sup>-/-</sup> B cells have increased proliferation and survival in response to both antigenic and inflammatory signals. B cells were isolated and purified from whole splenocytes of WT and I $\kappa$ B $\epsilon$ <sup>-/-</sup> cells using negative selection by CD43 magnetic beads. The separated B cells were stained with 1 nM CFSE and stimulated with either 10  $\mu$ g/ml IgM or 10  $\mu$ g/ml LPS. At designated time points, B cells were harvested and stained with 5  $\mu$ g/ml 7-AAD and analyzed for proliferation and death using flow cytometry. **(A)** B cells from I $\kappa$ B $\epsilon$ <sup>-/-</sup> mice displayed increased proliferation in response to both IgM and LPS at each time point. **(B)** The increased number of proliferating I $\kappa$ B $\epsilon$ <sup>-/-</sup> B cells over that of WT B cells was measured using FlowJo software, and the cell numbers were graphed. **(C)** Diagram depicting the fcyton model. In this model, stimulated cells undergo death over time (Tdie<sub>0</sub>) or enter division (pF<sub>0</sub>, fraction entering division; Tdiv<sub>0</sub>, time to division). **(D)** CFSE-proliferation profiles of I $\kappa$ B $\epsilon$ <sup>-/-</sup> and WT B cells stimulated with either IgM or LPS were analyzed using FloMax, running the Pcyton model to predict pF<sub>0</sub>, Tdiv<sub>0</sub>, and Tdie<sub>0</sub> cells. The fraction of responding B cells (pF<sub>0</sub>) was greatly increased in both IgM- and LPS-stimulated I $\kappa$ B $\epsilon$ <sup>-/-</sup> B cells compared with WT B cells. **(E)** 7-AAD measurements of B cell death show that (Figure legend continues)

kinetics may account for the stimulus-specific phenotypes. Using a mathematical modeling approach, we sought to test the latter hypothesis. To begin, we summarized the known relative relationships between the two potential negative-feedback regulators  $\text{I}\kappa\text{B}\alpha$  and  $\text{I}\kappa\text{B}\beta$  in terms of their interactions with RelA- and cRel-containing dimers, their differential responsiveness to IKK-induced degradation, and the stimulus-specific dynamics of IKK activity (Fig. 4A). Interestingly, we found that, although NF- $\kappa\text{B}$ -responsive  $\text{I}\kappa\text{B}\alpha$  gene expression required RelA, NF- $\kappa\text{B}$ -responsive  $\text{I}\kappa\text{B}\beta$  gene expression could be mediated by either RelA or cRel (Supplemental Fig. 2). Next, we constructed a mathematical model with these parameters by adapting a previously established mathematical model (3) to include the cRel dimers and to recapitulate B cell-specific dynamic control of RelA- and cRel-containing dimers (Supplemental Mathematical Model Equations). Simulations of this model with the IgM-induced transient IKK activity showed that RelA:p50 dimer is barely affected by  $\text{I}\kappa\text{B}\beta$  deficiency; however, in response to LPS-induced long-lasting IKK activity, RelA:p50 remains hyperactivated at late time points (Fig. 4B, upper panels). In contrast, cRel:p50 was hyperactivated under both conditions (Fig. 4B, lower panels). By quantitating the time course at 24 h, the stimulus-agnostic effect on cRel and stimulus-specific effect on RelA are readily appreciated (Fig. 4C); in fact, this graph closely resembles the experimental results obtained biochemically (Fig. 3C).

These simulation results demonstrate that the kinetic argument is a sufficient explanation for the stimulus-specific phenotype seen in  $\text{I}\kappa\text{B}\beta$ -deficient B cells. We can summarize the kinetic argument as follows: in response to transient IKK signals,  $\text{I}\kappa\text{B}\alpha$  is capable of providing postinduction repression on its high-affinity target RelA:p50 but less effectively on its low-affinity target cRel:p50, which requires  $\text{I}\kappa\text{B}\beta$  for complete suppression. However,  $\text{I}\kappa\text{B}\alpha$ 's responsiveness to long-lasting IKK signals renders it effectively neutralized; thus, under these conditions,  $\text{I}\kappa\text{B}\beta$ , which has lower responsiveness, plays an important role for its high-affinity target cRel:p50 and for its low-affinity target RelA:p50. Interestingly, the single specificity of the  $\text{I}\kappa\text{B}\alpha$  negative-feedback loop for RelA:p50 and the dual specificity of the  $\text{I}\kappa\text{B}\beta$  negative-feedback loop for RelA:p50 and cRel:p50 are reflected in the dimer requirements for  $\text{I}\kappa\text{B}\alpha$  and  $\text{I}\kappa\text{B}\beta$  inducible expression (Supplemental Fig. 1). We note that, although reported kinetic relationships (Supplemental Table I) are consistent with this sufficiency argument, we cannot rule out that a stimulus-specific signaling pathway also plays a role in the described phenotype.

#### *$\text{I}\kappa\text{B}\beta^{-/-}$ B cells show increased expression of NF- $\kappa\text{B}$ target genes*

To further characterize the phenotype at the molecular level, we examined how the gene-expression programs induced by IgM or LPS were affected by the loss of  $\text{I}\kappa\text{B}\beta$ . To this end, we used high-throughput sequencing of polyA RNA isolated from WT and  $\text{I}\kappa\text{B}\beta^{-/-}$  B cells at 0, 2, 8, and 24 h following stimulation with either IgM or LPS. Sequence data were converted into transcriptome levels using CummRbund (36), and these were normalized to the WT 0-h time point. We selected for genes induced in WT B cells by  $\geq 2$ -fold by IgM and LPS in at least one time point. This resulted in 881 and 846 genes, respectively. We identified hyperinduced genes as those that showed a  $\geq 2$ -fold change in  $\text{I}\kappa\text{B}\beta^{-/-}$  B cell data at two time points compared with their WT counterparts, resulting in 56 and 106 genes, respectively (Fig. 5A).

To determine whether the identified hyperinduced genes are under the control of NF- $\kappa\text{B}$  dimers, we used the HOMER motif discovery software adapted to perform searches of the known NF- $\kappa\text{B}$  dimer motifs identified previously (29) and summarized in this article (Fig. 5B, top panel). We found enrichment for these  $\kappa\text{B}$  motifs within the promoter regions of genes hyperinduced in  $\text{I}\kappa\text{B}\beta^{-/-}$  B cells compared with controls that were not hyperinduced (Fig. 5B). Interestingly, we found that enrichment of the cRel:p50 motif in hyperinduced genes was statistically significant under both IgM and LPS conditions; however, RelA:p50 motif enrichment was statistically significant only when B cells were activated with LPS. These results reflect the biochemical data that showed that, although cRel:p50 is hyperactivated in  $\text{I}\kappa\text{B}\beta^{-/-}$  B cells under both IgM and LPS conditions, RelA:p50 hyperactivation occurs primarily in response to LPS (Fig. 3C).

#### *Increased expression of IL-6 mediates the enhanced proliferation of $\text{I}\kappa\text{B}\beta$ -deficient B cells*

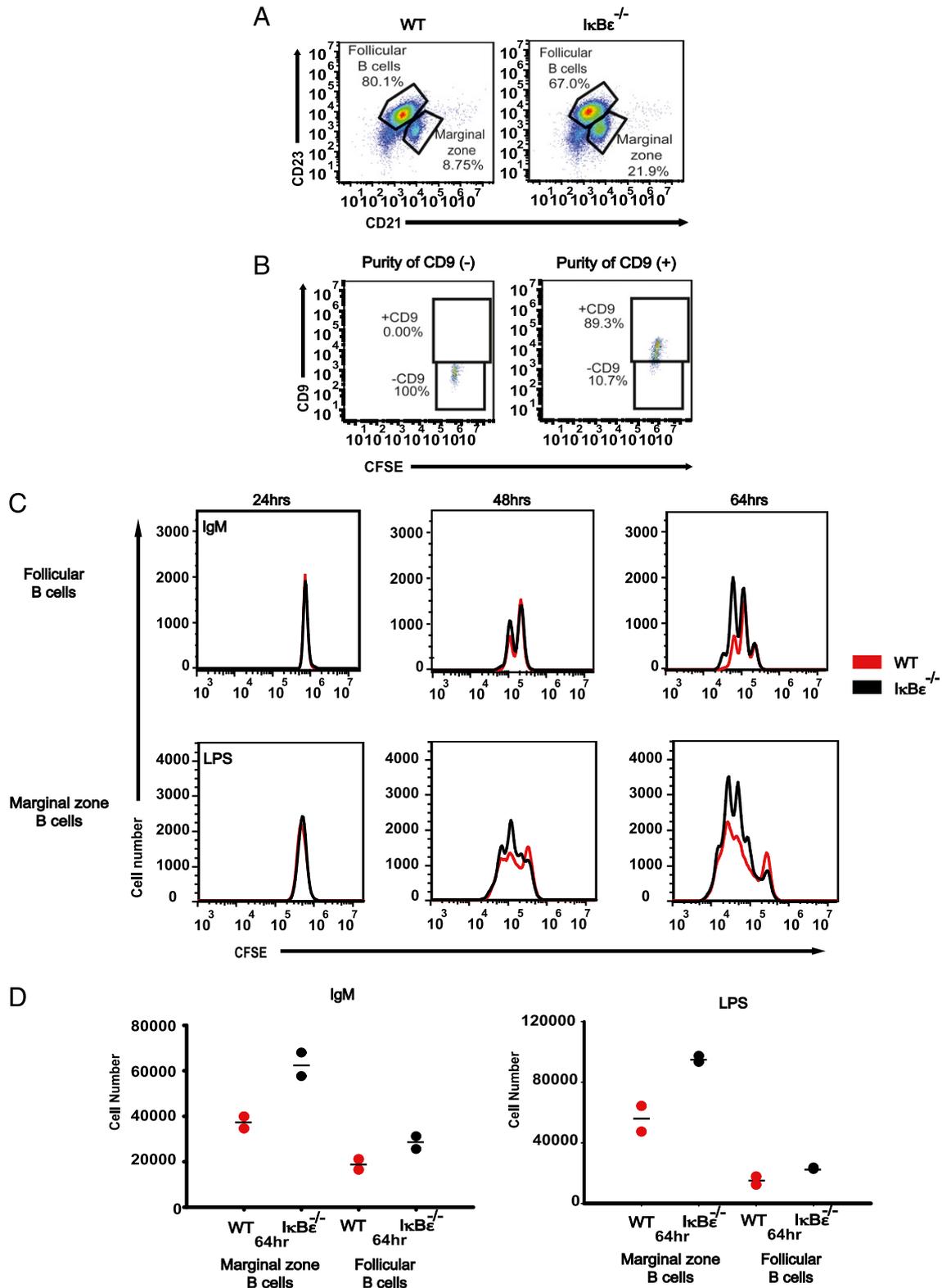
Inflammatory IL-6, which was initially discovered as a B cell-stimulating and differentiation factor, was among the NF- $\kappa\text{B}$  target genes that were hyperinduced in LPS-stimulated  $\text{I}\kappa\text{B}\beta^{-/-}$  B cells (Supplemental Table I). Subsequent studies of IL-6's effects on B cells found that it allows for increased proliferation, enhanced differentiation, and reduced apoptosis (37–40). The literature is unclear about whether IL-6's expression is cRel or RelA dependent (41–44). In our stimulation conditions, we found that IL-6 is robustly induced in response to LPS in WT cells both at the level of mRNA (Fig. 6A) and secreted cytokine measured in supernatants (Fig. 6B). As expected,  $\text{I}\kappa\text{B}\beta^{-/-}$  B cells show hyperinduction at both early and late times, with the strongest effect at the protein level being at the late time point of 24 h. Interestingly, cRel-deficient B cells showed no mRNA reduction at early time points (2 and 8 h), although a significant deficiency was observed at 24 h. Further, IL-6 hyperinduction appeared to be LPS specific, correlating with RelA hyperactivity and prior observations in other cell types that pointed to RelA-dependent expression (41–43). Together, these data suggest that IL-6 induction is triggered by RelA and then may be enhanced by cRel-containing dimers.

We next asked whether the enhanced proliferation of LPS-stimulated  $\text{I}\kappa\text{B}\beta^{-/-}$  B cells is potentially mediated by increased autocrine IL-6 costimulation of these cells. To test this hypothesis, we isolated B cells from WT or  $\text{I}\kappa\text{B}\beta^{-/-}$  mice and stimulated them with LPS in the presence of either 2  $\mu\text{g}/\text{ml}$  cytokine-neutralizing or 2  $\mu\text{g}/\text{ml}$  receptor-blocking Abs for IL-6. We used Abs neutralizing IL-1 $\alpha$  and IL-1 $\beta$  as controls, because these proproliferative cytokines were not found to be hyperinduced in our transcriptomic profiling. Effects on B cell proliferation were measured using CFSE staining. We found that Abs blocking IL-6 signaling had little effect on B cell expansion from WT mice; however, using B cells from  $\text{I}\kappa\text{B}\beta^{-/-}$  mice, we found a reduction in the B cell expansion to almost WT levels (Fig. 6C). Neither of the control Abs had an effect on the LPS-triggered expansion of WT or  $\text{I}\kappa\text{B}\beta^{-/-}$  B cells. These findings suggest that the increased production of IL-6 is responsible, at least in part, for the increased proliferative capacity of  $\text{I}\kappa\text{B}\beta^{-/-}$  B cells.

## Discussion

In this study, we identified  $\text{I}\kappa\text{B}\beta$  as a key negative-feedback regulator of cRel-containing NF- $\kappa\text{B}$  dimers in B cells, which limits

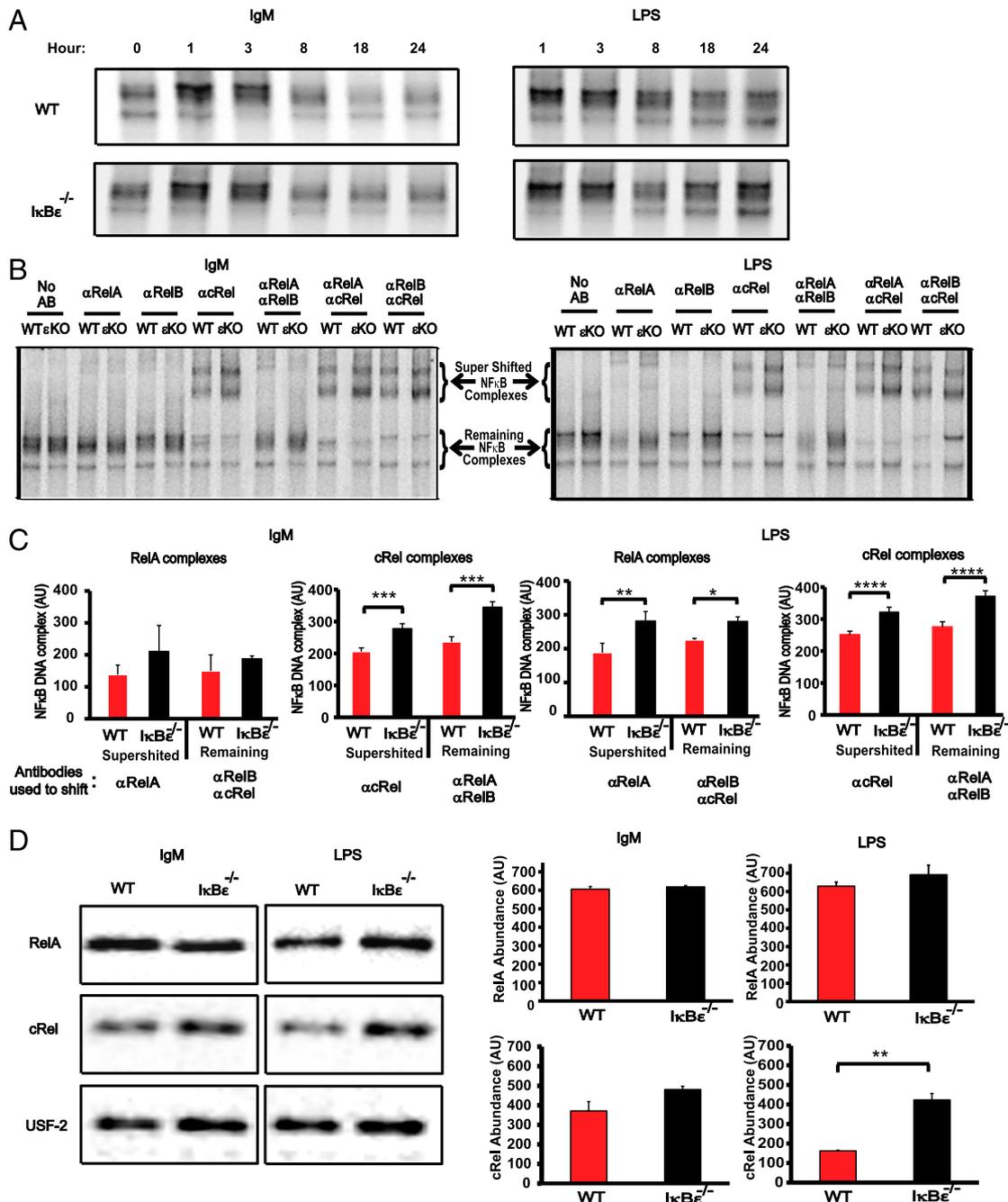
an increased percentage of B cells in the WT population undergo apoptotic death compared with  $\text{I}\kappa\text{B}\beta^{-/-}$  B cells. (F) The percentage of 7AAD<sup>+</sup> B cells from several experiments was measured using FlowJo software, and the percentages of 7AAD<sup>+</sup> cells were graphed. Data in (A), (B), (D), (E), and (F) are representative of at least four independent experiments. \*\* $p < 0.01$ , \*\*\* $p < 0.005$ , \*\*\*\* $p < 0.001$ , unpaired  $t$  test.



**FIGURE 2.** FO and MZ  $I\kappa B\epsilon^{-/-}$  B cells show increased proliferation. FO and MZ B cell populations were analyzed from whole splenocyte populations by flow cytometry using anti-B220, anti-CD21, and anti-CD23. **(A)**  $I\kappa B\epsilon^{-/-}$  B cells were composed of 67% FO B cells and 21.9% MZ B cells, whereas WT B cells were composed of 80.1% FO B cells and 8.75% MZ B cells. **(B)** MZ and FO B cells were separated by FACS sorting of anti-CD9. Purity of the separated CD9<sup>+</sup> and CD9<sup>-</sup> B cell populations was between 90 and 100%. **(C)**  $I\kappa B\epsilon^{-/-}$  FO and MZ B cells showed increased proliferation over WT FO and MZ B cells. **(D)** The increased number of proliferating  $I\kappa B\epsilon^{-/-}$  B cells over that of WT B cells was measured using FlowJo software, and the cell numbers were graphed. All data are representative of two independent experiments.

B cell proliferation in response to mitogenic stimulation. In contrast to our understanding based on fibroblast studies, we found that  $I\kappa B\epsilon$  also plays a nonredundant role in limiting the ubiquitous

RelA-containing NF- $\kappa$ B, albeit in a stimulus-specific manner. This result led to two insights: first from a physiological perspective we found that limiting RelA activation is relevant for controlling

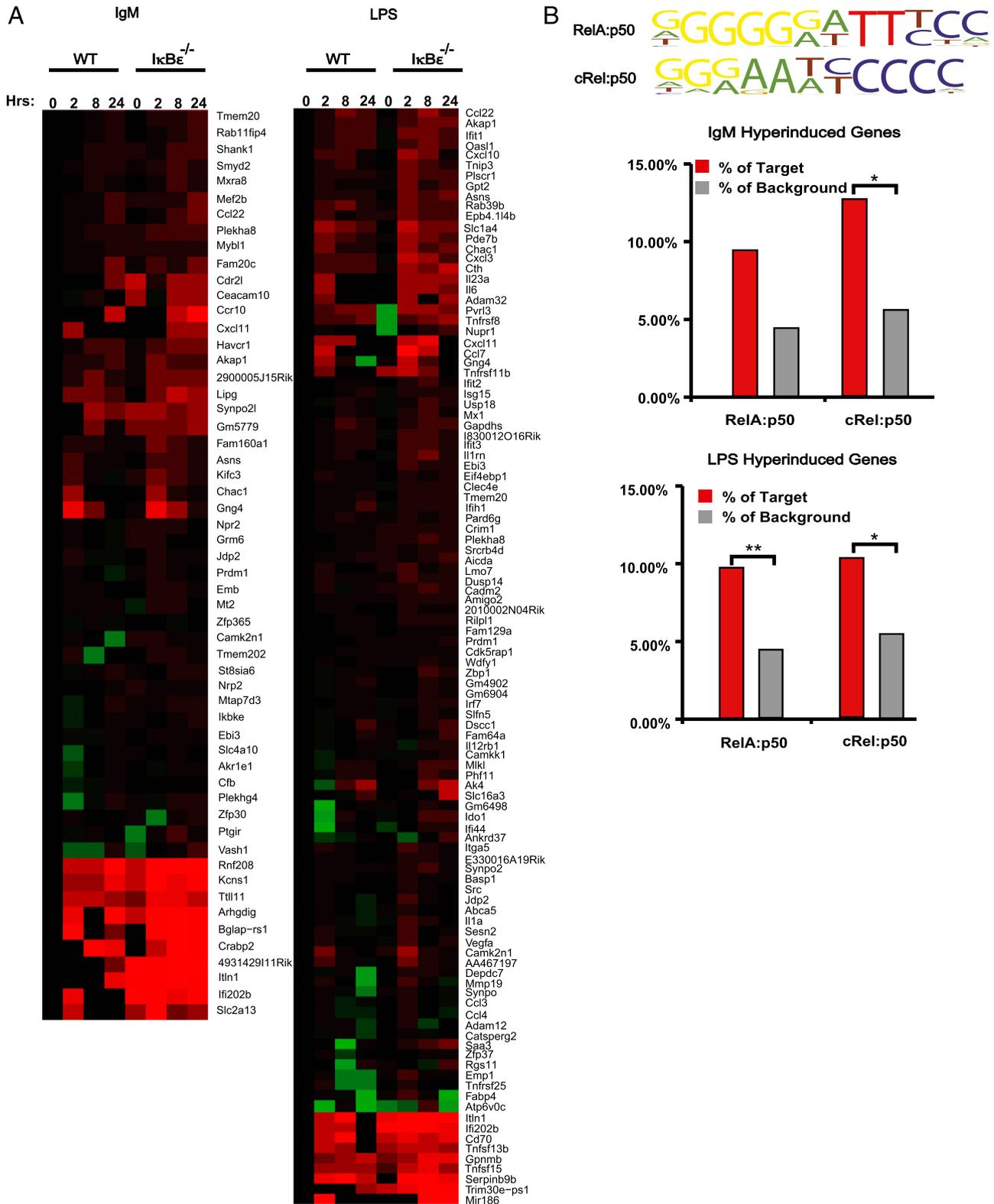


**FIGURE 3.** NF- $\kappa$ B activity is increased in  $I\kappa B\epsilon^{-/-}$  B cells. Purified B cells were collected and extracted into cytoplasmic and nuclear fractions at various time points. Nuclear extracts were tested for total NF- $\kappa$ B activity using EMSAs. **(A)**  $I\kappa B\epsilon^{-/-}$  B cells stimulated with either IgM or LPS exhibited increased NF- $\kappa$ B activity at 18 and 24 h following stimulation. **(B)** The 24-h nuclear extracts were incubated with Abs directed toward anti-RelA ( $\alpha$ RelA), anti-RelB ( $\alpha$ RelB), anti-cRel ( $\alpha$ cRel), as well as combinations of these Abs ( $\alpha$ RelA/ $\alpha$ RelB,  $\alpha$ RelA/ $\alpha$ cRel, and  $\alpha$ RelB/ $\alpha$ cRel). Following a 20-min incubation,  $^{32}$ P-labeled probe was added and allowed to incubate for an additional 15 min. The resulting samples were run on a 5% nonreducing acrylamide gel. **(C)** The resulting supershifts were quantitated using ImageJ software and graphed below each shift. Both IgM and LPS stimulation resulted in increases in cRel/p50 activity in  $I\kappa B\epsilon^{-/-}$  B cell extracts compared with extracts of WT B cells stimulated under the same conditions. LPS-stimulated  $I\kappa B\epsilon^{-/-}$  B cell extracts had increased RelA activity. **(D)** Nuclear Western blots for RelA and cRel were run and quantitated to determine whether the increased cRel activity observed in the supershifts was the result of increased RelA and cRel levels in the  $I\kappa B\epsilon^{-/-}$  B cells. Increased cRel protein levels were observed in the nuclear extracts from  $I\kappa B\epsilon^{-/-}$  B cells compared with WT B cell extracts. Similar levels of RelA were found in WT and  $I\kappa B\epsilon^{-/-}$  B cell nuclear extracts. Data shown in (A) are representative of two independent experiments. Data shown in (B), (C), and (D) are representative of three independent experiments ( $n = 3$ , error bars represent SD). \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.005$ , \*\*\*\* $p < 0.001$ , unpaired  $t$  test.

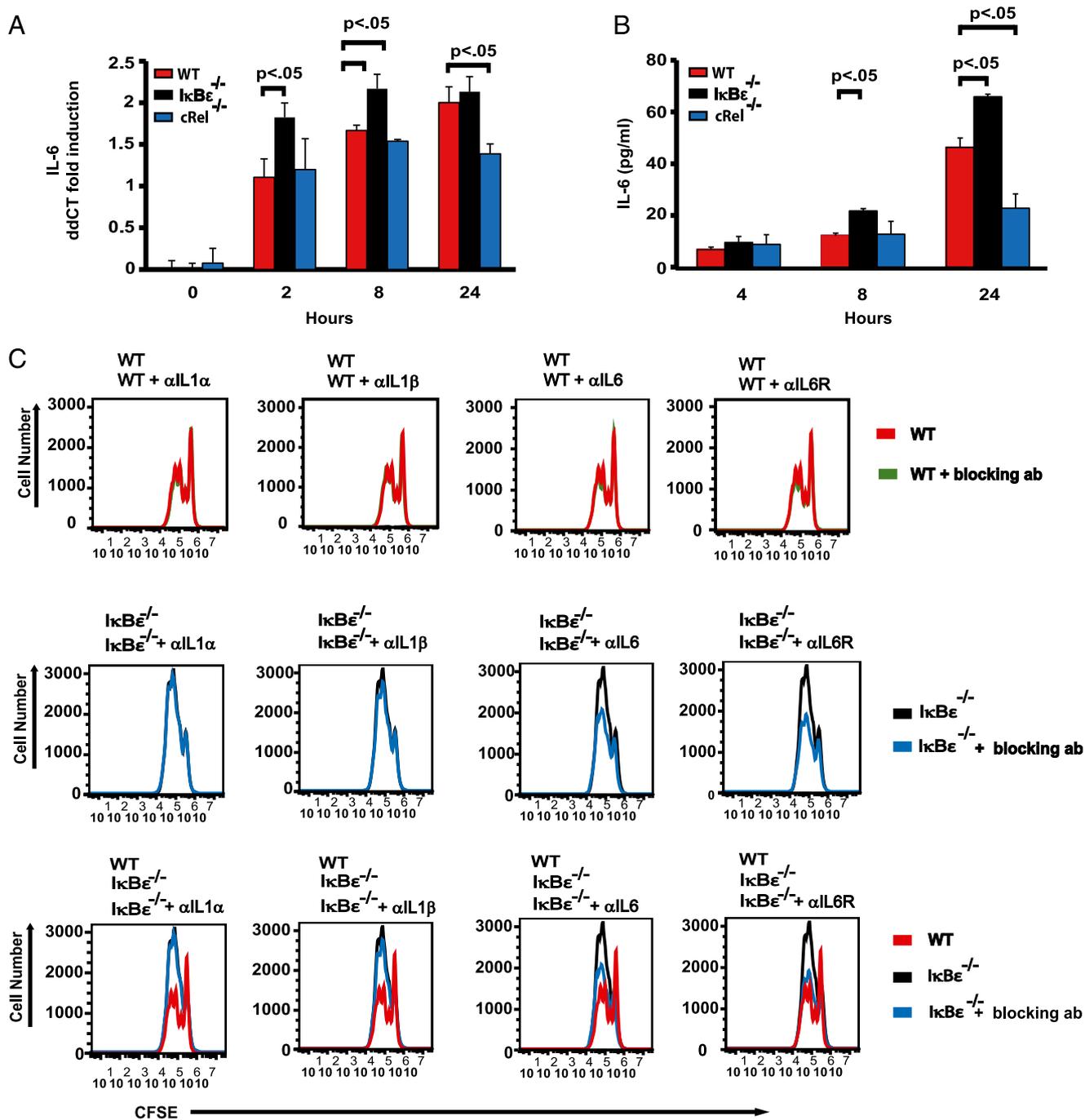
B cell expansion, because neutralizing the expression of the RelA target gene IL-6 mitigated the  $I\kappa B\epsilon$ -deficient phenotype. Second, considering the NF- $\kappa$ B system as a dynamic one, we conclude that the stimulus-specific functions of  $I\kappa B\epsilon$  negative feedback are based on kinetics rather than the engagement of a stimulus-specific mechanism or pathway.

Members of the  $I\kappa B$  protein family were found to preferentially bind different NF- $\kappa$ B members (22, 23, 45–47). Unlike  $I\kappa B\alpha$ , which is known to bind and regulate RelA-containing dimers,  $I\kappa B\epsilon$  was shown to bind with cRel homodimers and cRel:p50 heterodimers, although RelA-containing dimers may also be bound (22, 23). Interestingly, we found that these binding specificities are also





**FIGURE 5.** *IkBe*<sup>-/-</sup> B cells have an enrichment of NF- $\kappa$ B-dependent gene expression. Total RNA gene expression was obtained from RNA sequencing of both WT and *IkBe*<sup>-/-</sup> B cells at 0 h and after 2, 8, and 24 h of stimulation with IgM or LPS. Hyperinduced genes were identified as those having a 2-fold induction in at least one time point in WT B cells and an additional >2 fold increase in induction at two time points in the *IkBe*<sup>-/-</sup> B cells. (A) The genes identified as hyperinduced over WT were displayed as heat maps for IgM and LPS stimulation. (B) Motifs for the NF- $\kappa$ B dimers were loaded and used in Homer motif discovery software to search the promoter sequences of the identified induced and hyperinduced genes of *IkBe*<sup>-/-</sup> B cells for occurrences of the listed NF- $\kappa$ B motifs. The resulting genes containing one of these NF- $\kappa$ B motifs were graphed as percentages over the total genes found to be hyperinduced within the *IkBe*<sup>-/-</sup> samples. \**p* < 0.05, \*\**p* < 0.01.



**FIGURE 6.** Release of IL-6 is enhanced in I $\kappa$ B $\epsilon$ <sup>-/-</sup> B cells. B cells from WT and I $\kappa$ B $\epsilon$ <sup>-/-</sup> mice were isolated and stimulated with LPS. Supernatants and RNA extracts were collected at 0, 4, 8, and 24 h. **(A)** Quantitative PCR results for WT, I $\kappa$ B $\epsilon$ <sup>-/-</sup>, and cRel<sup>-/-</sup> B cells. IL-6 expression is enhanced by the loss of I $\kappa$ B $\epsilon$  and is partially dependent on cRel. **(B)** LPS stimulation of WT, I $\kappa$ B $\epsilon$ <sup>-/-</sup>, and cRel<sup>-/-</sup> B cells. I $\kappa$ B $\epsilon$ <sup>-/-</sup> B cells have increased cytokine release of IL-6 compared with WT and cRel<sup>-/-</sup> B cells, as assessed by ELISA. The loss of cRel reduced the amount of IL-6 released. **(C)** Neutralizing Abs against the cytokines IL-1 $\alpha$ , IL-1 $\beta$ , and IL-6 or receptor-blocking Abs against IL-1 $\alpha$ , IL-1 $\beta$ , and IL-6R were used at a concentration of 2  $\mu$ g/ml in B cell-proliferation assays. Reduced proliferation of I $\kappa$ B $\epsilon$ <sup>-/-</sup> B cells only occurred in the presence of Abs directed against the individual cytokines or their receptors for IL-6. WT B cell proliferation is only slightly reduced in the presence of the IL-6 Ab. Data shown in (A) and (B) are representative of three independent experiments ( $n = 3$ , error bars represent SD). Data shown in (C) are representative of two independent experiments.

a known activator of B cell proliferation, as being misregulated in response to both BCR and TLR stimulation. Interestingly, we also identified the RelA:p50 dimer as being misregulated, albeit only in cells responding to LPS, and not BCR, stimulation. NF- $\kappa$ B dimers may be distinguished biochemically and with immunological tools; however, given their somewhat different binding sequence preferences, they also may be distinguished, to some degree, by the binding motifs present in downstream target genes. We used

this approach by identifying hyperinduced genes in the I $\kappa$ B $\epsilon$ <sup>-/-</sup> B cells using RNA sequencing and screening their regulatory regions using Homer motif discovery software modified to search for NF- $\kappa$ B dimer motifs identified by Bulyk and colleagues (29). Intriguingly, the cRel:p50 motif was statistically significantly overrepresented in hyperexpressed genes in response to both stimuli, but the RelA:p50 motif only achieved statistical significance in response to LPS. Thus, two lines of evidence support the

conclusion that NF- $\kappa$ B dimers under the control of I $\kappa$ B $\epsilon$  are a function of the initiating stimulation.

Given the established role of cRel in B cell expansion, we examined whether RelA misregulation is functionally relevant. The cytokine IL-6 is one LPS-specific misregulated target gene whose NF- $\kappa$ B binding site conforms to the RelA-dimer motif. Remarkably, both the direct neutralization of IL-6 and the blocking of IL-6R reduced I $\kappa$ B $\epsilon^{-/-}$  B cell proliferation to near WT levels, while only slightly affecting WT B cell proliferation. These results suggest that the autocrine stimulation of IL-6 is responsible, at least in part, for the enhanced proliferation of I $\kappa$ B $\epsilon^{-/-}$  B cells in response to LPS and that hyperactivation of RelA is indeed functionally relevant. Our investigation of the proliferative phenotype observed in IgM-stimulated I $\kappa$ B $\epsilon^{-/-}$  B cells found IL-6 expression to be lacking, yet the IgM-stimulated I $\kappa$ B $\epsilon^{-/-}$  B cells still displayed increased proliferation compared with WT B cells. This difference between IgM- and LPS-stimulated I $\kappa$ B $\epsilon^{-/-}$  B cells could be explained by the differences in the downstream-stimulation pathways of IgM and LPS. The RNA sequencing data displayed in our study (Fig. 5A) demonstrate that there is very little overlap of the genes upregulated by each pathway. We propose that stimulation with LPS leads to a proliferative phenotype that is dependent on IL-6 upregulation and stimulation, yet, in the case of IgM stimulation, upregulation of a different set of proliferative genes occurs.

How may the same negative-feedback regulator target different signal transducers in response to different stimuli? By adapting an established mathematical model of the NF- $\kappa$ B–signaling module to B cells, we showed in this study that a kinetic explanation is sufficient to account for the observations. Specifically, differences in the interaction parameters of I $\kappa$ B $\alpha$  and I $\kappa$ B $\epsilon$  with RelA and cRel dimers, in conjunction with I $\kappa$ B's differential responsiveness to IKK activities, whose temporal profiles are, in turn, stimulus specific, could reproduce the stimulus-specific control of the RelA dimer. Thus, the present study is an extension of previous work that demonstrated that kinetic differences between I $\kappa$ B $\alpha$  and I $\kappa$ B $\delta$  could impart them with stimulus-specific functions: although I $\kappa$ B $\alpha$  is critical for turning off NF- $\kappa$ B activity following transient activation signals, I $\kappa$ B $\delta$  limits NF- $\kappa$ B activity when the activation signals are long lasting (3). As our understanding of signaling systems improves and mathematical modeling is adopted more widely, we may expect to find an increasing number of examples in which consideration of the kinetics is critical for an understanding of the specificity of observed phenomena.

## Disclosures

The authors have no financial conflicts of interest.

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