B cell survival and development controlled by the coordination of NFκB family members RelB and cRel

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Running Title: cRel and RelB control B-cell development
Key Points

1. NFκB family members RelB and cRel are coordinately activated by BAFF and provide distinct survival signals.

2. *In vivo* and *in vitro* B cell developmental defects are observed when both RelB and cRel are deleted.

Abstract

Targeted deletion of BAFF causes severe deficiency of splenic B cells. BAFF-R is commonly thought to signal to NIK-dependent non-canonical NFκB RelB. However, RelB-deficient mice have normal B cell numbers. Recent studies showed that BAFF also signals to the canonical NFκB pathway, and we found that both RelB and cRel are persistently activated, suggesting BAFF-signaling coordinates both pathways to ensure robust B cell development. Indeed, we report now that combined loss of these two family NFκB members leads to impaired BAFF-mediated survival and development *in vitro*. While single deletion of RelB and cRel was dispensable for normal B cell development, double knockout mice displayed an early B cell developmental blockade and decreased mature B cells. Despite disorganized splenic architecture in Relb<sup>−/−</sup> cRel<sup>−/−</sup> mice, generation of mixed-mouse chimeras established the developmental phenotype to be B cell intrinsic. Together, our results indicate that BAFF signals coordinate both RelB and cRel activities to ensure survival during peripheral B cell maturation.
Introduction

B cell development originates in the bone marrow, where hematopoietic stem cell precursors commit to the B cell lineage and immunoglobulin heavy-chain gene rearrangements occur\(^1,2\). If rearrangement is successful, differentiation into the transitional B cell compartment occurs. Cells which generate functional B cell antigen receptors (BCR) eventually leave the bone marrow and migrate to the spleen to complete their maturation process\(^3,4\). The first B cells to arrive are referred to as transitional-1 B cells (T1)\(^5,6\). T1 B cells are still subject to negative selection, where strong antigenic signals lead to apoptosis. In later transitional stages, some of the transitional B cells (T2) are allowed to develop into either follicular mature (FO) B cells, which can recirculate in the periphery, or marginal zone B cells (MZ), which remain largely sessile\(^7,8\).

The B cell activation factor receptor belonging to the TNF superfamily (BAFF-R, BR3) provides critical survival signals to all splenic B cell subsets. Targeted deletion of BAFF ligand or BAFF-R results in a partial block at the T1 to T2 transition, resulting in severe deficiency of mature B cells\(^9,10\). BAFF initiates the non-canonical NF\(\kappa\)B pathway via TRAF3, resulting in the stabilization of NIK and activation of a NEMO-independent IKK1 kinase complex. This mediates p100 processing, and nuclear translocation of RelB:p52 dimers\(^11\). Recent human studies have shown that patients with germline mutations in \(NFKB2\) have immunodeficiency. In some of the patients, there is a loss of B cells\(^12-14\). It is likely that some of these B-cell developmental defects in the patients result from impaired BAFF-R signaling, due to their non-processable p100. BAFF has also been reported to activate the canonical NF\(\kappa\)B pathway\(^15,16\). Gene-targeted deletion of
NFkB1 (p50), the primary binding partner of RelA and cRel, results in defective survival of B cells in response to BAFF\textsuperscript{17}. And while neither $cRel^{-/-}$ nor $Rela^{-/-}$ mice show a phenotype in B cell numbers, doubly-deficient B cell precursors fail to develop the full mature subsets\textsuperscript{18}. This raises the question of whether the non-canonical NFκB pathway and RelB play any role at all in safeguarding B-cell development. However, we note that RelA/cRel-deficiency is also known to diminish RelB expression and non-canonical signaling\textsuperscript{19-22}. The same considerations apply to interpreting other severe knockouts of the canonical pathway such as B cell-specific NEMO or IKK2 knockouts\textsuperscript{23,24}. The fact that the $Nfkb1^{-/-}Nfkb2^{-/-}$ mouse shows a phenotype similar to BAFF/BAFFR-deficient mice (unlike either single mutant) suggests that both pathways may be redundant\textsuperscript{11}. However, studies of a compound knockout of the two transcriptional activators that mediate canonical and non-canonical pathways, respectively, have not been reported. Here, we show that only cRel and RelB show persistent activation in response to BAFF, and we therefore examine the physiological consequence of their deletion singly or in combination. We find that both provide survival signals, albeit via distinct gene expression programs, and that these complement each other, such that only the doubly deficient mouse shows severe B cell developmental deficiencies. Deficiencies in mature B cell subsets are based not solely on survival defects but also a block in differentiation block at the transitional T1 stage that is cell autonomous and can be observed in an \textit{ex vivo} differentiation assay.
Materials and Methods

Cell isolation and culture

Spleens were harvested from C57BL/6 wild type, cRel⁺/-, Relb⁺/-, Relb⁺/-cRel⁻/- or IκBα⁻/-IκBε⁻/-cRel⁻/-TNF⁻/- mice. B cell isolation performed by anti-CD43 (Ly-48) microbeads and separated on LS column (Miltenyi Biotec) as in previous studies. Purity was confirmed to be between 92-95% (data not shown). Marginal Zone and Follicular B Cell Isolation Kit (Miltenyi Biotec) was used for separation of follicular B cells from whole splenocytes, with a purity of 83-87% CD23⁺ follicular B cells (Figure S1A). Complete media consisted of RPMI-1640, 10mM HEPES, 1mM Sodium Pyruvate, 1mM non-essential amino acids, 0.055mM β-mercaptoethanol, 100 units Penicillin/Streptomycin and 0.3mg/ml glutamine.

FACS analysis of survival and B cell development

Purified B cells were cultured in complete media with or without 50ng/ml recombinant mouse BAFF / BLyS / TNFSF13B (R&D systems 2106BF). At indicated time points, B cells were collected and stained with 7AAD (Invitrogen A1310). Cells were analyzed for survival using a C6 Accuri flow cytometer (BD Biosciences). B cell development was obtained from single-cell suspensions of spleens incubated with fluorescently labeled antibodies for 30 min at 4°C in staining buffer (PBS with 0.5% BSA or 2.5% FCS). Data were collected on a FACSCalibur or LSR II flow cytometer (BD Biosciences). FACS analyzed using FlowJo software (Tree Star, Inc.). Antibodies used in this study from eBioscience (unless specified) included reagents specific for the following: CD21 (7G6),...
B220 (RA3-6B2), CD23 (B3B4); Ly5.1 (A20), Ly5.2 (RA3-6B2), CD93, (AA4.1), IgD (11-26) and IgM (115-096-020; Jackson Immunoresearch).

**Immunohistochemistry**

Spleens from wild type or Relb<sup>+/−</sup>cRel<sup>−/−</sup> mice were frozen in OCT compound above liquid nitrogen and kept at -80°C. 5 micron sections were cut from frozen spleens, fixed in cold 4% paraformaldehyde and allowed to dry. Sections were then H&E stained or subjected to immunofluorescent labeling. Sections were blocked with 5% FBS in PBS and incubated with anti-Moma1, anti-CD3 FITC, and anti-B220 APC (eBioscience) for three hours or O/N. Washed sections were mounted with Fluoromount-G (eBioscience) and Images were captured on a CRI Nuance Multispectral Imaging (Caliper, Hopkinton, MA) system attached to a Nikon E800 fluorescent microscope.

**Bone marrow chimeras**

Donor bone marrow cells were isolated from femurs of wild type, cRel<sup>+/−</sup>, Relb<sup>−/−</sup> or Relb<sup>−/−</sup>cRet<sup>−/−</sup> mice, mixed in a 50 / 50 ratio, and injected (~5x10<sup>6</sup> cells intravenously per mouse) into 2- to 3-month-old C57BL/6 or C57BL/6 x B6.SJL-Ptprca F1 recipients. Before injection, recipient mice were lethally irradiated (1,000 rads). Chimeric mice were analyzed 8 weeks after bone marrow reconstitution. Origin and composition of lymphoid cells was determined by Ly5.1 and Ly5.2 markers.

**In vitro B cell differentiation**
In vitro differentiation was performed as previously described\textsuperscript{29}. Briefly, bone marrow cells were cultured in complete IMDM with 20ng/ml IL-7 for 3–4 d to enrich for IgM\(^+\) transitional B cells. Cells were then washed twice with PBS to remove IL-7 and plated with 50ng/ml recombinant murine BAFF. On subsequent days, cells were stained with anti-B220, IgM, and IgD antibodies to determine maturation.

Biochemical analysis

Electrophoretic mobility shift assays (EMSAs) were conducted as described previously\textsuperscript{22,30}, and modified to include antibodies specific for RelA (Santa Cruz SC372), RelB (SC226), cRel (SC71), or a combination thereof. For EMSAs focusing on cRel-DNA binding activity, nuclear extracts were pre-incubated with RelA and RelB antibodies for 15min on ice prior to addition of radiolabeled probe to ablate their specific DNA binding activities. Similarly, nuclear extracts were pre-incubated with RelA and cRel antibodies or RelB and cRel antibodies when RelB- or RelA-DNA binding activity was the focus, respectively (see Figure 1B). Other antibodies used for immunoblotting were Akt (Cell Signaling Technology, CST#4691), phospho-Akt (Ser473) (CST#4060), I\(\kappa\)B\(\alpha\) (SC371), I\(\kappa\)Be (SC7155), \(\alpha\)–Tubulin (SC26), NFkB1/p50 and NFkB2/p52 (Dr. Nancy Rice) and Bclxl (Abcam, #ab2568).

Transcriptome analysis

Total RNA was extracted from 50ng/ml BAFF-stimulated wild type B cells. mRNA was isolated from 2\(\mu\)g total RNA using oligo (dT) magnetic beads and fragmented at high
temperature using divalent cations. cDNA libraries were generated using the IlluminaTruSeq kits and quantitation was performed using the Roche Light Cycler 480. Sequencing was performed on Illumina's HiSeq 2000, according to the manufacturer’s recommendations and prepared for RNA sequencing analysis by the Broad Stem Cell Research Center core facility at the University of California, Los Angeles. Reads were aligned to the mouse mm10 genome and RefSeq genes\textsuperscript{31,32} with Tophat\textsuperscript{33}. Cufflinks CummRbund\textsuperscript{34} was used to ascertain differential expression of genes. Gene differential FPKMs were obtained from the cuffdiff program in the Tuxedo RNA-seq analysis suite. RNAseq data is deposited in NCBI’s Gene Expression omnibus and accessible through GEO Series accession number (GSE62559).
Results

**BAFF induces persistent NFκB RelB and cRel activities that have complementary roles in mediating B cell survival**

BAFF is known to engage three BLyS family receptors, namely B cell maturation Ag (BCMA), a transmembrane activator and calcium-modulating / cyclophilin ligand-interacting protein (TACI) and BAFF-R (also known as BR3 or BLYS receptor)\(^{35}\), and to activate several signaling pathways including the canonical and non-canonical NFκB pathways, whose transcriptional effectors are RelA/cRel and RelB, respectively (Figure 1A).

We examined which NFκB transcription factors are activated by BAFF in murine splenic B cells. We used a modified EMSA procedure in which antibodies are used to ablate two of three activation-domain containing NFκB family members (Figure 1B). Stimulation with BAFF ligand resulted in activation of RelA, cRel, and RelB containing dimers, but the timecourses reveal that only cRel and RelB show persistent activation: RelA activity peaks at one hour and returns to basal levels within 8 hours. In contrast, cRel and RelB activities are not short lived, peaking at ~3 hours and persisting beyond 24 hours (Figure 1C). Repeated experimental analysis showed that these conclusions were statistically significant (Figure 1D).

Numerous studies have investigated which signaling pathways mediate BAFF’s physiological functions, and these genetic knockout studies are summarized in Figure 1E. Of the three known BAFF-binding receptors, expression of BCMA is not observed in naive B cell populations, and TACI deletion actually results in enhanced B cell numbers.
and splenomegaly whereas, BAFF-R deletion phenocopies BAFF deletion in resulting in a near loss of peripheral B cells. BAFF activates both canonical and non-canonical signaling branches of the NFκB system, yet, deficiencies in only canonical components (e.g. NEMO, RelA/cRel) have been reported to result in defective B cell development (Figure 1E). However, the canonical pathway, and RelA in particular, is known to enable non-canonical signaling via NFκB2 and Relb expression (Figure 1A), suggesting that phenotypes observed in severe canonical deficiencies might be mediated by defects in non-canonical signaling.

As both RelB and cRel dimers are activated persistently by BAFF, we focused on examining their roles in mediating its key function, survival. Whole splenic B cells were isolated from Relb−/−, cRel−/−, and Relb−/−cRel−/− mice, and stimulated with BAFF for up to four days. In the in vitro survival assay, RelB-deficient B cells respond poorly to 70 hours of BAFF stimulation, and cRel deletion also displays a minor defect in the BAFF response; however compound deletion of both cRel and RelB resulted in a statistically more severe phenotype (Figure 2A) than single deletion compared to wild type. We observe a similarly severe survival defect in a homogeneous population of mature follicular (CD23+) B cells isolated from the spleen of wild type and Relb−/−cRel−/− mice (Figure 2B). These data suggest that BAFF signaling coordinates the functions of both canonical and non-canonical NFκB transcription factors to promote survival.

Next we asked whether the cRel and RelB mediated their complementary functions by inducing the same or different sets of survival genes in B cells. We undertook RNA-sequencing (RNA-seq) of BAFF stimulated mature follicular (CD23+) wild type, cRel−/−,
and Relb−/−cRel−/− B cells. Here, we identified 517 genes upregulated in BAFF-stimulated wild type B cells, 289 genes of which were substantially reduced in Relb−/−cRel−/− B cells (Figure 2C). Of these, 127 genes were largely cRel-dependent, as they show lower BAFF-induced expression in both cRel−/− or Relb−/−cRel−/−B cells, and 162 were identified as RelB-dependent in the context of cRel-deficiency, and may thus be described as potential RelB target genes. Interestingly, gene ontology analysis focusing on biological process terms identified a large number of receptor-associated and mitochondrial anti-apoptotic regulators within the cRel-dependent cluster, which metabolic regulators were primarily identified in the RelB/cRel-dependent cluster (Figure 2D, E). These data suggest that coordination of cRel and RelB activities by BAFF serves the expression of complementary sets of genes that ensure B cell survival. Confirmatory RT-qPCR showed that Bcl2 family members A1 and Bcl-xL required only cRel but not RelB for their expression, and the reduction is mRNA expression also resulted in a reduction in Bcl-xL protein (Figure S2).

**Compound deletion of RelB and cRel results in severe peripheral B cell developmental defects.**

As RelB and cRel are critical for BAFF-mediated survival functions *in vitro*, we examined B cell development *in vivo* in mice lacking RelB, cRel, or both. Using an established FACS strategy we confirmed that there is no defect in peripheral B cell populations in cRel−/− mice and only a modest decrease in Relb−/− mice. However, we found a significant loss of mature B cells and an increase in the transitional compartment
in double knockout mice (Figure 3A), while T cell numbers were normal (Figure S3).

Distinguishing mature B cell subsets, we confirmed previous reports that Relb⁻/⁻ spleens were deficient in the marginal zone (MZ) B cells population³⁸ but now found that the double knockout mice generate significantly fewer mature follicular (FO) B cells (Figure 3B, Table 1).

Dissection of transitional B cell populations revealed an increased proportion of transitional T1 but not T2 cells (Figure 3C). T1 B cells are the initial class of B cells which gives rise to the mature populations³⁹. In addition, we examined the B cell maturation in the bone marrow of the double knockout and found no discernible defect compared to wild type controls (Figure S4). The increase in T1 B cells found in the double knockout mouse, suggested to us that RelB and cRel are not only required for the survival of developing B cells but also for peripheral B cell development per se. While the Relb⁺/⁻ cRel⁻/⁻ developmental phenotype is not as severe as that caused by BAFF deficiency, the block at the T1 stage is reminiscent⁴⁰. Further, histological analysis corroborated these findings: in the Relb⁻/⁻ cRel⁻/⁻ spleen, the white pulp, while present, was not as dense (Figure 3D). The reduction in white pulp may be caused by reduced numbers of B cells in the spleen, as our FACS analysis suggested, or it may be a consequence of improper homing of the lymphocytes causing them to be dispersed throughout the spleen.

To address this, we investigated the organization of B cells using immunofluorescence. Frozen sections of splenic tissue were stained with anti-CD-3 and anti-B220 to identify the T-cell zone and B-cell zone, respectively. MOMA-1 (which stains MZ metallophilic macrophages) outlined the follicular rim surrounding the B cell rich zone. In agreement
with our FACS data, the presence of B cells was greatly diminished in the \( \text{Relb}^{-/-}\text{cRel}^{-/-} \) spleen, as shown by the staining of B220 (red) (Figure 3E). We also observed that the characteristic ring-like structure of MOMA-1 staining (blue) is consistently less organized in the double knockout mouse, suggestive of a defective splenic architecture, which may\(^{41,42} \) or may not contribute to the B cell development phenotype in the mutant mice.

**B cell intrinsic developmental defect in vivo.**

We studied whether the B cell developmental phenotype is in fact B cell intrinsic by generating mixed-bone marrow (BM) chimeras, where bone marrow derived hematopoietic stem cells from both wild type and mutant donor mice were injected into a lethally irradiated wild type or mutant recipient mice. To distinguish cells deriving from either donor, we utilized the CD45 congenic marker system in which either donor cells expressing the CD45.1 or CD45.2 variant, while recipient mice were CD45.1.2 (Supplemental Figures 5A-B).

Within this experimental system, we found that \( \text{Relb}^{-/-}\text{cRel}^{-/-} \) (CD45.2) hematopoietic stem cells were compromised in their development even when wild type recipients provided a normal milieu of architecture and soluble factors (Figure 4A-B) akin to observations made in cRel and RelB deficient animals (Figure 3A-C). Again, the transitional B cell populations are higher than the wild type controls, whereas FO populations were severely decreased and MZ B cells were undetectable (Figure 4A-B). As a reference, we also generated chimeras with RelB and cRel single knockout bone
marrow cells, with no observable phenotype (Figure S5C-D). When wild type and Relb−/− cRel−/− donor bone marrow stem cells were injected into lethally irradiated Relb−/−cRel−/− recipient mice, wild type donor stem cells were able to mature normally from T1 to T2, FO, and MZ B cells, while Relb−/−cRel−/− donor stem cells matured poorly (similar to when they were in wild type host) (Figure 4C-D). These data provide strong evidence that the B cell developmental phenotype of Relb−/−cRel−/− mice is in fact cell intrinsic, and not caused by improper splenic architecture or cytokine milieu.

**Relb−/−cRel−/− progenitors are defective in BAFF-mediated B cell development in vitro**

A true cell intrinsic phenotype is expected to be recapitulated in an in vitro differentiation assay in which all non-intrinsic components are held equal. We employed an in vitro B cell development assay system described previously11,43. In this system, transitional B cells are generated from bone marrow progenitors in the presence of IL-7. The cytokine is then washed away, then recultured with BAFF ligand (Figure 5A). Expression of B cell markers CD23 and CD21 has been reported to be regulated by BAFF 44. Further, FACS analysis suggested that RelB plays a role in CD21 expression (Figure 3B), thus we used IgD and B220 as our primary developmental markers in these analyses. Exposure of wild type BM cultures to BAFF for 4 days increased the fraction of IgD+ cells relative to untreated controls, demonstrating BAFF's capacity to enhance B cell development in vitro. In contrast, BAFF stimulation failed to increase the percentage of IgD+ cells present in Relb−/−cRel−/− BM cultures (Figure 5B-C). Even in the absence of BAFF, the numbers of IgD+ cells were consistently lower in mutant cultures compared to wild type.
In fact, the reduction in B cell numbers in this assay may be dominated by a BAFF-dependent survival defect (Figure 5D). Together, these experimental results further illustrate the necessity of RelB and cRel in BAFF-mediated B cell maturation.

**Deregulating NFκB accelerates splenic B cell development**

Given that NFκB RelB/cRel activities are required for B-cell maturation past the T1 stage (Figure 3), we asked whether elevated NFκB might be sufficient to accelerate B-cell maturation. Compound deficiency of IκBα and IκBε leads to elevation of both constitutive and stimulus-responsive NFκB activity\(^{45}\), inflammatory gene expression and neonatal death\(^{46}\). We restored viability by introducing compound cRel and TNF deficiencies, leaving RelA and RelB deregulated, and examined the B-cell compartment. Although the total splenocytes count lower in *IκBα/−IκBε/−cRel/−TNF/−* mice than *wild type* controls, the proportion of B220+ cells is relative normal (~40-50%). Interestingly, in contrast to hypo-NFκB activity in *Relb/−cRel/−* mice, hyper-NFκB activity in *IκBα/−IκBε/−cRel/−TNF/−* mice leads to reduced T1 cell numbers and a preponderance of MZ B cells (Figure 6). Though the reduced B-cell numbers also indicate restrictions in bone marrow-developmental events, these observations are indicative of accelerated splenic B-cell development.

**Discussion**
For over a decade, numerous reports have focused on the characterization of non-canonical NFκB signaling in response to BAFF signaling pathway\(^\text{17,24,47}\), yet only mutants of the canonical pathway have BAFF\(^{+/−}\)-like phenotypes. In this study, we report that the canonical NFκB effectors cRel and RelA are activated following BAFF stimulation. Further, while RelA activation is transient, cRel activation is persistent and thus mimics the activation profile of RelB, the well-known effector of the non-canonical pathway.

Indeed, both cRel and RelB play a role in BAFF functions \textit{ex vivo}, as either singly-deficient B cell showed reduced BAFF-responsive survival. Remarkably, however, these survival functions appear to be mediated by different gene-expression programs, with cRel being required for the expression of known pro-survival genes A1 and Bcl-xL\(^{16,48}\), but \textit{Relb}\(^{−/−}\)-B-cells showing no defects in their expression. Whereas unbiased transcriptome analysis of RelB-deficient B-cells is compromised by the confounding secondary effects of systemic inflammation in the \textit{Relb}\(^{−/−}\) mouse\(^{49}\), \textit{Relb}\(^{+/−}\)\textit{cRel}\(^{−/−}\) mice, which do not show an inflammatory phenotype, allowed us to identify the RelB-dependent gene expression program in the context of cRel-deficiency. The complementary nature of the cRel- and RelB-dependent expression programs manifests itself in the fact that double deletion leads not only in a more severe B cell survival defect \textit{ex vivo} (Figure 2), but also \textit{in vivo} (Figure 3).

Further, we found that double deletion of RelB and cRel results in a block in splenic B cell development at the transitional 1 to 2 stage. These mutant mice are not only deficient in mature cells but possess a two-fold increase in the percentage of transitional B cells,
and almost four-fold enhancement in the population of T1 cells. This developmental blockade at T1 to T2 stage mimics the phenotype of BAFF knockout mice, though the defect is not quite as severe, suggesting a role for RelA, or a NFκB-independent mechanism. This developmental defect is B cell intrinsic, as Relb<sup>−/−</sup>cRel<sup>−/−</sup> bone marrow stem cells failed to develop normally in mixed-bone chimeras and wild type hematopoietic stem cells are capable of proper B cell development in lethally irradiated Relb<sup>−/−</sup>cRel<sup>−/−</sup> mice.

How does BAFF coordinately activates cRel and RelB? Three TNF receptor superfamily members bind BAFF, the B cell maturation Ag (BCMA), a transmembrane activator and calcium-modulating / cyclophilin ligand-interacting protein (TACI) and BAFF-R (also known as BR3 or BLYS receptor)<sup>41</sup> with BAFF-R and TACI being expressed on peripheral B cell subsets<sup>50</sup>. TACI activates canonical NFκB signaling<sup>51</sup>, but TACI-deficient mice show no defect on B cell development and B cells are capable of degrading IκBα in response to BAFF<sup>15</sup>. Our results are consistent with BAFF-R triggering both canonical, IκBα-mediated NFκB signaling, and also non-canonical, IκBδ-mediated activation of RelA and cRel. As a key survival receptor, BAFF-R signaling maintains the B cell pool. In contrast, TACI acts as a negative regulator of B cell homeostasis, indirectly regulating peripheral B cell numbers by competing for the amount of BAFF available for signaling through BAFF-R<sup>41,52</sup>.

CD21 expression is reduced in the absence of RelB, and further decreased in the combined absence of RelB and cRel (Figure 3B). CD21 is known to be controlled by NFκB-p52<sup>53,54</sup>. Considering our data, CD21 is probably controlled by RelB:p52 complex
during development, and cRel:p52 to a lesser extent (possibly providing compensation).

Given that CD21 is essential in humoral immune response\textsuperscript{55,56}, it is likely RelB-deficient B cells will have impaired response to certain T-dependent antigens, especially complement-tagged antigens\textsuperscript{57-59}. Further functional studies are needed to address this.

Previous studies suggested that NF\textsubscript{κB} regulates BAFF-R expression\textsuperscript{60,61}, though none of the NF\textsubscript{κB} knockout studies cited in Figure 1E had examined BAFF-R expression. We examined BAFF-R expression in two ways: replicate RNA-seq did not show an expression defect in \textit{Relb}\textsuperscript{-/-}\textit{cRel}\textsuperscript{-/-} cells over wild type controls (Figure S6A) thereby not corroborating transcriptional control by RelB/cRel. However, by FACS, BAFF-R staining was reduced (Figure S6B) and BAFF-responsive AKT phosphorylation (indicative of PI3 kinase activation was lower also (Figure S6C). Together, this suggests that the phenotype of \textit{Relb}\textsuperscript{-/-}\textit{cRel}\textsuperscript{-/-} B-cells may in part be reinforced by a reduction in BAFF-R surface expression. As BAFF-R is transcribed at the normal rate, this reduction is not a direct consequence of RelB/cRel-deficiency, but probably rather a consequence of cell-biological deficiencies that hinder B-cell maturation from the T1 stage. Further, though the loss of cRel and RelB reduces the amount of p100, as observed elsewhere\textsuperscript{62}, BAFF-responsive non-canonical NFkB2/p100 degradation remained intact (Figure S6C).

The coordinate activation of cRel and RelB renders BAFF’s physiological responses relatively robust, as single knockout show little \textit{in vivo} defect. At the same time, cell-context-specific control of the two branches of NFkB signaling and activation of their respective gene-expression programs may allow for fine tuning of BAFF’s broad physiological functions in controlling survival, B cell maturation, cell growth and even
proliferation. Matching quantitative studies of signaling strength and dynamics, to gene expression programs and cell biological level analyses may lead to a better understanding how BAFF functions \textit{in vivo} and enable its pharmacological manipulation in the clinical setting.
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Authorship Contributions

JA, YL, AH, MA, MD, and AG designed the research and interpreted data. JA and YL performed all B cell biochemistry. JA, DO and MD performed IF. JA and YL performed in vitro B cell development. JA, HB, and JD-T carried out RNAseq analysis. JA, EY, MA and AG designed and made bone marrow chimeras. JA, YL and AH wrote the manuscript.

Conflict of interest

The authors declare no competing financial interests.
References


Figure legends

Figure 1. BAFF-R stimulation triggers persistent RelB and cRel activity to control in vitro survival functions. A. BAFF, which binds TACI and BAFF-R, is a key regulator of peripheral B cell maintenance. BAFF-R signaling functions occur primarily in an a NIK-dependent, non-canonical NFκB manner; mediated through RelB activity, while TACI triggers NEMO-dependant canonical NFκB. B. Identification of activated NFκB species in B cells stimulated with BAFF for 24hr by supershift analysis. C. RelA, RelB and cRel EMSA (see methods) of B cells are utilized to reveal their respective activity kinetics following BAFF-R perturbation. D. Quantification of RelA, RelB and cRel activities of BAFF stimulated primary B cells derived from EMSA; Naïve is resting, unstimulated B cells, early and late activity correspond to activities following 1-5 hours and 24+ of BAFF stimulation respectively. Representative of ≥ 4 experiments; * p<0.05; ** p<0.01. E. Summary table of known NFκB deficient mouse models and its effect on B cell populations. Grey boxes denote a B cell developmental defect. EMSA for (B-C) representative of at least three experiments.

Figure 2. RelB and cRel coordinate together to provide proper BAFF-mediated survival signals in vitro. A. FACS plots of in vitro survival assay of whole splenic wild type, Relb−/−, cRel−/−, and Relb−/−cRel−/− B cells stimulated with BAFF ligand for 70hr. Numbers represent the percentage of live cells (7AAD−) found in culture. Graphical representation of the FACS plots (right) n=3. B. FACS plots of in vitro survival assay of
mature follicular B cells (CD23+) wild type and Relb<sup>−/−</sup>cRel<sup>−/−</sup> B cells stimulated with BAFF for 40hr. C. RNAseq analysis from BAFF stimulated CD23<sup>+</sup> wild type, cRel<sup>−/−</sup>, and Relb<sup>−/−</sup>cRel<sup>−/−</sup> B cells at the indicated time points. 517 genes were upregulated in BAFF-stimulated follicular B cells. 289 of these showed substantial expression defect in B cells lacking both cRel and RelB (middle and bottom panel). Of these, 127 showed expression defects even in the single cRel knockout (middle panel); 162 showed expression defects only in the Relb<sup>−/−</sup>cRel<sup>−/−</sup> double knockout (bottom panel). D. cRel-dependent genes protect cells against cell death. Gene Ontology analysis identifies distinct process terms for cRel-dependent vs. RelB/cRel-dependent gene clusters. Whereas RelB/cRel-dependent clusters are significantly associated with terms describing metabolic processes, the cRel-dependent cluster shows over-representation of negative regulation of cell death/apoptosis. * p<0.05; ** p<0.005; *** p<0.001. Also see Figure S1. E. List of representative cRel-dependent and RelB/cRel-dependent genes identified as “negative regulators of cell death” and “metabolic process”, respectively, by gene ontology analysis.

**Figure 3. Compound deletion of RelB and cRel results in developmental block at transitional one stage.** A-C. FACS analysis of peripheral B cell development in whole splenic extracts from wild type, Relb<sup>−/−</sup>, cRel<sup>−/−</sup>, and Relb<sup>−/−</sup>cRel<sup>−/−</sup> mice. Identification of mature (B220<sup>+</sup>AA4.1<sup>−</sup>) and transitional B cells (B220<sup>+</sup>AA4.1<sup>+</sup>). Mature B cells (B220<sup>+</sup>AA4.1<sup>−</sup>) are further classified into mature follicular (FO) B cells (CD21<sup>+</sup>IgM<sup>+</sup>) and mature marginal zone (MZ) B cells (CD21<sup>hi</sup>IgM<sup>+</sup>). Transitional B cells
(B220^+ AA4.1^+) are subdivided into transitional one (T1) B cells (CD23^-IgM^+) and transitional two (T2) B cells (CD23^-IgM^+). Scatter plots are graphical representation of FACS plots: wild type (•) Relb^-/^-cRel^-/^- and Relb^-/^-cRel^-/^- ( ).

D. Histological analysis of splenic sections taken from wild type and Relb^-/^-cRel^-/^- mouse using hematoxylin and eosin stain (H&E).

E. In vivo analysis of mature B cell development in double knockout mouse using immunofluorescence of frozen splenic sections stained with 1) anti-CD3 FITC, 2) anti-B220 APC, and 3) anti-MOMA-1 Alexa Fluor® 450. * p<0.05; ** p<0.005; *** p<0.001. Also see Figure S2.

**Figure 4. B cell developmental defect displayed in Relb^-/^-cRel^-/^- mouse is B cell intrinsic.** A and C. Schematic for the generation of CD45.1 wild type and CD45.2 Relb^-/^-cRel^-/^- mixed bone marrow chimeras. Bone marrow stem cells from CD45.1 wild type and CD45.2 Relb^-/^-cRel^-/^- are mixed in a 50/50 ratio, and then injected into a lethally irradiated CD45.1.2 wild type or Relb^-/^-cRel^-/^- mouse. B and D. Graphical plots of percentages of specific subsets relative to total B-cells in indicated mixed bone marrow chimeras, as described above. The gating strategy used in the analysis is depicted in Figure 3. Representative FACS plots see Figure S3. For B (n=5). For D (n=2). * p<0.05; ** p<0.005; *** p<0.001.

**Figure 5. Relb^-/^-cRel^-/^- B cell progenitors fail to respond to BAFF mediated developmental signal in vitro.** A. Schematic of in vitro B cell differentiation system. B. Representative FACS plots of B220 and IgD expression in transitional B cells at the
beginning (day 0) and end (day 4) of culture with or without BAFF. C. Mean frequency of B220⁺IgD⁺ (T2-like) B cells following four days of culture as described in B. Numbers represent frequencies of live B220⁺ B cells in indicated gates. D. Survival of in vitro cultured B cells. Live cells were identified by gating out 7AAD⁺ population. Fold survival calculated from initial B cell population (day 0). For FACS plots (n=4).

Figure 6. Deregulated NFκB activity in IκBα⁻/⁻IκBε⁻/⁻cRel⁻/⁻TNF⁻/⁻ leads to reduction in T1 cells and increased MZ B cell generation. FACS analysis of peripheral B cell development in whole splenic extracts from wild type, cRel⁺/⁺, Relb⁺/⁺cRel⁺/⁺, and IκBα⁺/⁺IκBε⁺/⁺cRel⁺/⁺ TNF⁺/⁺ mice. The gating strategy used in the analysis is depicted in Figure 3. Numbers represent percentage of denoted subsets as proportion of total B cells. Representative FACS results of 3 experiments shown.
Table 1. Summary of peripheral B cell development in wild type, Relb\textsuperscript{−/−}, cRel\textsuperscript{−/−}, and Relb\textsuperscript{−/−}cRel\textsuperscript{−/−} mice

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>wild type</th>
<th>Relb\textsuperscript{−/−}</th>
<th>cRel\textsuperscript{−/−}</th>
<th>Relb\textsuperscript{−/−}cRel\textsuperscript{−/−}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Cells</td>
<td>75.4 ±17</td>
<td>131 ±15\textsuperscript{**}</td>
<td>76.1 ±8.3</td>
<td>104 ±25\textsuperscript{*}</td>
</tr>
<tr>
<td>Total B cells</td>
<td>51.3 ±6.9</td>
<td>39.8 ±8.6\textsuperscript{*}</td>
<td>41.6 ±4.9</td>
<td>29.1 ±7.8\textsuperscript{****}</td>
</tr>
<tr>
<td>Mature</td>
<td>32.8 ±6.7</td>
<td>23.3 ±7.1\textsuperscript{*}</td>
<td>30.1 ±4.4</td>
<td>14.3 ±4.2\textsuperscript{*}</td>
</tr>
<tr>
<td>Transitional</td>
<td>9.28 ±1.9</td>
<td>7.85 ±1.7</td>
<td>9.86 ±2.5</td>
<td>12.7 ±2.7\textsuperscript{*}</td>
</tr>
<tr>
<td>Transitional 1</td>
<td>3.00 ±1.2</td>
<td>3.17 ±1.2</td>
<td>3.58 ±0.94</td>
<td>6.30 ±2.0\textsuperscript{**}</td>
</tr>
<tr>
<td>Transitional 2</td>
<td>5.42 ±0.64</td>
<td>5.21 ±2.0</td>
<td>5.54 ±1.3</td>
<td>6.48 ±2.5</td>
</tr>
<tr>
<td>Marginal Zone</td>
<td>3.1 ±1.1</td>
<td>0.067 ±0.052\textsuperscript{****}</td>
<td>3.2 ±0.071</td>
<td>0.31 ±0.34\textsuperscript{****}</td>
</tr>
<tr>
<td>Follicular</td>
<td>25.3 ±6.3</td>
<td>22.4 ±5.1</td>
<td>24.1 ±4.4</td>
<td>12.5 ±3.6\textsuperscript{***}</td>
</tr>
</tbody>
</table>
Table 2. Summary of B-cell subset number in NFκB-deleted mice.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Transitional 1</th>
<th>Transitional 2</th>
<th>Marginal Zone</th>
<th>Follicular</th>
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<tbody>
<tr>
<td>Wild type</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Baff&lt;sup&gt;-/-&lt;/sup&gt; Baffr&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>10,40</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tac&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>36</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
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<tr>
<td>Relb&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>38,49</td>
<td>+++</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>cRel&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>63</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Rela&lt;sup&gt;-/-&lt;/sup&gt; Tnf&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>64</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
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<tr>
<td>Nfkb1&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>65</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>Nfkb2&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>66-68</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ikk1&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>69-71</td>
<td>+++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Ikk2&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>23</td>
<td>+++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Nemo&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>23,24</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Rela&lt;sup&gt;-/-&lt;/sup&gt; cRel&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>18</td>
<td>++</td>
<td>+</td>
<td>-</td>
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<tr>
<td>Nfkb1&lt;sup&gt;-/-&lt;/sup&gt; Nfkb2&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>11,67</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
A B

C

D

E

Figure 1
**Figure 2**

A. Whole splenic B cells

- **(-) BAFF**
  - Wild type
  - Relb<sup>-/-</sup>
  - cRel<sup>-/-</sup>
  - Relb<sup>-/-</sup>cRel<sup>-/-</sup>

- **(+) BAFF**
  - Wild type
  - Relb<sup>-/-</sup>cRel<sup>-/-</sup>

B. CD23<sup>+</sup> B cells

- **(-) BAFF**
  - Wild type
  - Relb<sup>-/-</sup>cRel<sup>-/-</sup>

- **(+) BAFF**
  - Wild type
  - Relb<sup>-/-</sup>cRel<sup>-/-</sup>

C. 7AAD cell count

- **(-) BAFF**
  - Wild type
  - cRel<sup>-/-</sup>

- **(+) BAFF**
  - Wild type
  - cRel<sup>-/-</sup>

D. Developmental maturation

- **cRel-dependent**
  - cRel/RelB-dependent

E. Gene expression

- **Negative regulation of cell death**
  - Vegfa, Bcl2l1, Cdkn1a, Rag1, Bcl2a1

- **Metabolic process**
  - Pkl, Pipam1, Tpp1, Aidroc, Hk2, Ldh, Pga1, Pkm, Gapdh

---

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Figure 3

A

B

C

D

E

**wild type**  | **Relb**-/**-**  | **cRel**-/**-**  | **Relb**-/**cRel**-/**-**

**Total cells x 10^6**

- Mature Immature
- Marginal zone Follicular
- Transitional 1 Transitional 2

**NS**

**Moma1 (macrophages)**
**CD3 (T cells)**
**B220 (B cells)**
Figure 4
**Figure 5**

(A) Schematic diagram showing the experimental setup: Day (-4) with IL-7, Day 0 with +/- BAFF, and Day 4 with FACS analysis.

(B) Flow cytometry plots showing the relative survival of IgD+ B cells in wild type and Relb^-/-cRel^-/- mice at Day (-4), Day 0, and Day 4 for (-) BAFF and (+) BAFF conditions.

(C) Bar chart comparing the percentage of IgD+ B cells in wild type (wt) and Relb^-/-cRel^-/- mice under (-) BAFF and (+) BAFF conditions. The graph shows a significant difference with asterisks indicating statistical significance.

(D) Bar chart showing the relative survival (%) of IgD+ B cells in wild type (wt) and Relb^-/-cRel^-/- mice under (-) BAFF and (+) BAFF conditions. The graph indicates a significant difference with asterisks.
B cell survival and development controlled by the coordination of NFκB family members RelB and cRel

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