UNIVERSITY OF CALIFORNIA, SAN DIEGO

Distinct and overlapping roles of canonical and non-canonical NFkappaB signals in B cell biology

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by

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2013

The Dissertation of Jonathan Virgilio Almaden is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

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2013

DEDICATION

To my parents and two sisters for their unending love and support

To my lovely wife for giving me everything and anything I needed to undergo this journey through graduate school (including our two lovely children)

To Shaoxian Sun, your enthusiasm for science gave me the desire to return to school, and your training allowed me to complete this thesis

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LIST OF ABBREVIATIONS

BCR	B cell receptor
BAFF-R	B cell activating factor receptor
T1	Transitional-1 B cells
T2	Transitional-2 B cells
MZ	Marginal Zone
FO	Follicular Mature
IF	Immunofluorescence
H&E	Hematoxylin and eosin stain
EMSA	Electrophoretic mobility shift assay APRIL
TACI	Transmembrane activator and calcium modulator and cyclophilin ligand interactor
TRAIL	TNF-related apoptosis-inducing ligand
NFκB	Nuclear Factor kappaB
TNF	Tumor necrosis factor
BLyS	B lymphocyte stimulator
BCMA	B cell maturation antigen

LPS	Lipopolysaccharide
RelB	v-rel reticuloendotheliosis viral oncogene homolog B
IκBα	Inhibitor of kappaB alpha
IκBβ	Inhibitor of kappaB beta
IκBδ	Inhibitor of kappaB delta
IκBε	Inhibitor of kappaB epsilon
IKKα	Inhibitor of kappaB kinase alpha
IKKβ	Inhibitor of kappaB kinase beta
IKKγ	Inhibitor of kappaB kinase gamma
IKKε	Inhibitor of kappaB kinase epsilon
WT	Wild type
KO	Knockout
GC	Germinal Center
PAMPS	Pathogen associated molecular patterns
CSR	Class-Switch Recombination
IF	Immunofluorescence

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ABSTRACT OF THE DISSERTATION

Distinct and overlapping roles of canonical and non-canonical NFkappaB signals in B cell biology

by

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The task of the adaptive immune system is to respond and clear pathogens. Tailoring responses based on pathogen type and retain memory of the pathogenic encounter for subsequent challenges are also requisite. For this, millions of individual B lymphocytes in the developing immune system

express unique antigen receptors, creating a vast repertoire of potential antigen-recognition specificities. Regulation of lymphocyte selection and development is tightly controlled, as aberrant selection can result in autoreactive or diminished responses, thus pathogenesis.

The NFκB transcription factor family is essential for lymphocyte proliferative responses during immune activation, but there is also evidence that it may play a role in lymphocyte development. The NFκB family comprises dimers of 5 related subunits; p50, p52, p65 (RelA), cRel, and RelB, which control gene expression by binding κB elements in the regulatory regions of target genes. Signals from antigen and innate immune receptors cause rapid, transient activation of NFκB through the NEMO/IKK-dependent, so called "canonical" signaling pathway, resulting in immediate degradation of IκBs and nuclear translocation of pre-existing RelA and cRel containing dimers. A second "alternative" NFκB pathway has been described downstream of TNFR family members such as BAFF-R which results in persistent activation of NFκB via kinase NIK.

NFκB was initially discovered in B cells. Since then the many components of the NFκB system have been identified and a mathematical model recapitulates the functioning of this dynamical system in fibroblasts, yet in B lymphocytes studies have generally focused on an individual NFκB component and systems level understanding remains lacking. This thesis describes our attempts to provide a predictive understanding of the functional

role of canonical and non-canonical NFκB regulation in the context of B cell development and activation. Chapter one gives an overview of mechanism by which B cells are produced and maintained along with current understanding of NFκB roles within this progression. Chapter two reports on NFκB effectors required for B lymphocyte maturation and development. Chapter three describes how BAFF-R stimulation contributes to BCR-triggered proliferation via two distinct NFκB signaling mechanisms provided by the non-canonical pathway. Finally, chapter four summarizes our findings in the context of current and future studies in the field.

CHAPTER 1:

Introduction

Overview of B cells

B cells are responsible for several immunological functions, considered primarily as a positive regulator of immune responses due to its ability to produce antibodies. Upon infection, B cells become activated and produce these compounds that attach and neutralize foreign antigens circulating in our bloodstream. Following the initial illness, some the B cells will remain. These long-lived memory B cells stay in our system in a ready-mode to quickly recognize and attack the returning virus or bacteria. This facility is a double-edged sword, as aberrant antibody function contributes to the pathogenesis of autoimmune related diseases.

B cells are restricted through stringent homeostatic controls, shown by the relatively constant lymphocyte pool size. A growing literature indicates that limitations in both pro- survival resources and physical space define these homeostatic constraints (Crowley et al., 2008; Miller et al., 2006). Within the B lymphocyte lineage, the B cell antigen receptor (BCR) plays a critical role in determining B cell survival (Torres et al., 1996)(Lam et al., 1997). In addition, the B cell-activating factor belonging to the TNF family (BAFF), aka B lymphocyte stimulator (BLyS) family of cytokines and receptors has also proved to be an equally vital determinant of B cell homeostatic regulation (Mackay et al., 2003; Moore et al., 1999). Signaling through these receptors activates distinct NFκB dimers, a transcription factor family essential for the survival of developing B cells in the spleen, and for the antigen dependent

activation and proliferation of lymphocytes. Within this overview we will examine the origins of the peripheral B cells subsets, and the functions of NFκB in the control of development, homeostasis, and activation of B cells.

Origins of immature B cells in the bone marrow

B cells in the bone marrow originate from a pool of multipotential stem cells. These newly formed B cells within the bone marrow are subject to various selection pressures which are meant to purge the nascent B cell pool of autoreactive and therefore potentially pathogenic B cells, as well as direct differentiation of immature B cells into functionally discrete peripheral B cell compartments. Prior to its exodus to the periphery, the development of these immature B cells from B lineage stem cells must first overcome discrete milestones.

In mammals, early B cell development occurs in the bone marrow, where these sequential differentiation stages are measured according to surface marker expression and immunoglobulin (Ig) gene rearrangement position (1–8). Decades of extensive and remarkable work achieved to describe this process are reviewed elsewhere (Cooper, 2002; Rolink and Melchers, 1996;Rolink et al., 1999), which we will discuss briefly (Table 1.1).

B cell development in the bone marrow can be classified into six to seven sequential subsets designated Fractions A, B,C,C',D,E,F by Hardy, Hayakawa, and colleagues (Hardy et al., 1991; Hardy and Hayakawa, 1991). B cells first rearrange their heavy-chain genes during the pro-B stage (Fr. A - C') (Osmond, 1986)(Cancro, 2004; Hardy and Hayakawa, 2001). All of these pro-B cell subsets express B220 and CD43. After successful Ig heavy chain rearrangement, CD43 surface expression is downregulated as cells progress

to fraction D (pre-B) cells. This stage also marks the first appearance of pre-B cell receptor (pre-BCR) on the cell's exterior, along with the Igα and Igβ(Hardy et al., 2007).

Cells within fraction D rearrange Ig light chains. Immature marrow B cells (Hardy fraction E) arise from those which have successfully undergone light-chain rearrangement. It is at this phase where B cells first express a complete BCR, which follows an exodus from the bone marrow to the spleen for continued maturation. These immature lymphocytes are the immediate progenitors of most mature peripheral B cells.

Developing B cells are first tested for self-tolerance in the bone marrow subsequent to the expression of the newly arranged BCR. Cells which interact with self-antigen are either deleted, undergo receptor editing, or become anergic, depending on the affinity and the physical form of the antigen encountered (Goodnow, 1996; Melchers, 2006). Also, it is at this stage where BAFF binding is evident, marking the initial period at which this cytokine can influence homeostatic processes.

Table 1.1.Cell surface expression on bone marrow B cell subsets.

Osmond	Hardy	IgG gene status	Surface Expression
Early Pro	A	Germline	$CD19^{-}B220^{lo}CD43^{+}HSA^{-}AA4.1^{+}$
Intermediate Pro	B	D-J_H	$CD19^{+}B220^{lo}CD43^{+}HSA^{+}AA4.1^{+}$
Late Pro	C	V_H-DJ_H	$CD19^{+}B220^{lo}CD43^{+}HSA^{+}AA4.1^{+}$
Large Pre	C'	Cyt mu, cyt mu	$CD19^{+}B220^{lo}CD43^{-}$ $HSA^{+}AA4.1^{+}preBcR^{+c}$
Small Pre	D	V_k-J_k	$CD19^{+}B220^{lo}HSA^{+}sIgM^{-}$
Immature	E	$sIgM^{+}sIgD^{-}$	$CD19^{+}B220^{lo}HSA^{+}$

Adapted from (Cancro, 2004)

Peripheral B cell development

Immature B cells are have successfully edited their BCRs by completing new Igκ or Igλ light chain rearrangements and immature B cells that are weakly or not self-reactive can mature further, through brief transitional (T1 and T2) B cell stages, into mature follicular or marginal zone B cells (Table 1.2)(Figure 1.1). The proportion of these B cells are few, as a large majority of immature B cells remain strongly self-reactive and can undergo receptor editing or clonal deletion (Gay et al., 1993; Hartley et al., 1991;Tiegs et al., 1993). As such, functionally immature B cells are defined by their short half-lives, their dominance during early phases of reconstitution of the peripheral B cell pool, and their propensity to undergo apoptosis rather than to proliferate following BCR engagement (Norvell et al., 1995).

The observation that B cells enter the periphery as immature B cells arising from the BM was first published by Allman et al. (Allman et al., 1992; Allman et al., 1993). The term transitional B cell was subsequently introduced by Carsetti et al. describing recently migrated immature splenic B cells in transition from the immature to the mature B cell compartments and a target of BCR-mediated selection (Carsetti et al., 1995). Carsetti and colleagues first proposed that immature or ‘transitional’ B cells in the adult spleen may be subdivided into two distinct subsets (T1 and T2) (Loder et al., 1999).

Transitional B cells comprise 5–15% of the adult splenic B cell population (Allman et al., 1992). They are subdivided into unique categories

based on location within and out the periphery, surface marker expression and physical properties. Early studies on immature peripheral B cells used the heat-stable antigen (HSA), also known as CD24, to distinguish the immature ($CD24^{hi}$) from mature ($CD24^{lo}$) splenic B lymphocytes (Allman et al., 1992) Carsetti and colleagues (Loder et al., 1999) used additional cell-surface expression profiling (CD21, CD23, and IgD) to further subdivide the $CD24^{hi}$ transitional B cell population into two developmental subsets. The first of these transitional B cells (T1) have yet to acquire the ability to recirculate and are found in the bone marrow and spleen. Newly generated B cells that have entered the follicles, acquired cell surface IgD and CD23 and the ability to recirculate, but still carry markers of immaturity, are known as T2 B cells (Allman et al., 2001).

In vivo transfer experiments using bulk-sorted cell populations suggested that T1 cells give rise to T2 cells and mature B cells and that T2 cells can give rise to mature B cells (Loder et al., 1999). The lifespan of T1 and T2 cells is very short (3–4 days), and T1 cells generate T2 cells within 48hr in vivo (Loder et al., 1999). T2 cells can develop into phenotypically mature follicular B cells after 1–2 days in vivo (Loder et al., 1999) and 2–3 days in vitro (Batten et al., 2000; Su and Rawlings, 2002). These results support a model in which splenic B cell development proceeds sequentially from T1 to T2 to mature B cell stages.

In a separate report, an alternative method of defining immature splenic B cells was described (Allman et al., 2001) using surface marker CD93 / AA4.1 which would present a contrasting definition of T1 and T2 B cells. In general, T1 B cells classified by both phenotyping strategies are largely compatible. A critical discrepancy exists in the later stages: in the HSA scheme, a percentage of the T2 populations are cycling. In contrast, utilizing the AA4.1-based scheme, none of the transitional subsets are dividing. Additionally, a third transitional stage was also described in which $AA4.1^{+}$ T3 immature B cells are the intermediary subset of B cells which can give rise to follicular mature B cells (Allman et al., 2001). However, compelling reports have arisen advocating that it is not an actual milestone towards maturation, but in fact a dead-end sub-population of anergic transitional B cells which failed to advance to the next stage of development (Merrell et al., 2006)(Teague et al., 2007). With this in mind, we focused on the two step classification based upon the HSA scheme which defines T1($CD21^{lo}CD23^{lo}CD24^{hi}IgM^{hi}IgD^{lo}$) and T2 ($CD21^{hi}CD23^{hi}CD24^{hi}IgM^{hi}IgD^{hi}$).

Following movement into the follicles, T2 cells can diverge into two distinct mature subsets: marginal zone (MZ) B cells or follicular (FO) B cells. Both of which wield distinct functions as components of the adaptive and innate immune system. The cell fate by which a T2 cell will pursue has been extensively reviewed here (Allman and Pillai, 2008; Pillai and Cariappa, 2009). Briefly, examination of various genetic models involving the deletion or

inactivation of genes encoding key components of the BCR signal transduction network has given rise to the hypothesis that low level BCR signaling provokes marginal zone B cell development, while relatively strong signals emitted from BCR favors the development of follicular B cells.

FO B cells (also called B-2 cells) represent most mature B cells, developing throughout life from transitional B cells (Allman et al., 1993). They reside in follicles, adjacent to T cell zones. This arrangement allows activated follicular B cells and activated T helper cells to migrate towards each other and interact at the interface between these two areas. Follicular B cells are therefore particularly well suited to participate in T cell-dependent immune responses to protein antigens (Rajewsky, 1996). Further, recent evidence proposes that follicular B cell populations can be separated into two distinct classes: FO-I and FO-II. Follicular Type I IgM^{low} B cells make up the majority of cells in the follicular B cell population, while type II comprise one third of the long-lived recirculating B cell pool (Cariappa et al., 2007).

A distinct T2 population ($IgM^{hi}IgD^{hi}CD21^{hi}CD1d^{hi}CD23^{hi}$), capable of cycling, yet cells only seen in the spleen and not in bone marrow, blood, or lymph nodes was identified by Loder et al. (Loder et al., 1999). Originally thought to be FO precursors; this notion was later proved incorrect. Examination of aiolos null mice revealed that this particular T2 B cells to be absent, yet FO B populations were plentiful. Analogous to this observation, is this subset is preserved in the Btk deficient mice, yet FO populations are lost.

Due to this, they are presently assumed to be a direct descendant of MZ B cells, coined marginal zone precursors (MZP) (Pillai et al., 2005; Saito et al., 2003).

Marginal zone B cells are phenotypically, functionally, and anatomically distinct from follicular B cells and plays a major role in responses to T cell independent (TI) antigens. Whereas follicular B cells are freely recirculating lymphocytes, MZ B cells are sessile, and not part of the recirculating pool in rodents. MZ B cells are self-replenishing, and localized at the border between the white and red pulp. They are rapidly recruited in T-cell independent antibody responses raised against blood-borne pathogens. The MZ B cell repertoire has low-level self-reactivity, which maintains MZ B cells in a pre-activated state, enabling them to respond rapidly to pathogens (Lopes-Carvalho and Kearney, 2004).

Table 1.2.Cell surface phenotypes of splenic B cell populations.

B cell Subset	Surface Expression
Transitional T1	$CD23^{-}$ $AA4^{+}$ $sIgM^{high}$ $sIgD^{-}$ HSA^{high} $CD62L^{-}$ $CD21/35^{low}$
Transitional T2	$CD23^{+}$ $AA4^{+}$ $sIgM^{high}$ $sIgD^{high}$ HSA^{high} $CD62L^{+}$ $CD21/35^{low}$
T2-MZP	$CD23^{+}$ $AA4^{-/low}$ $sIgM^{high}$ $CD1d^{+}$ $sIgD^{high}$ HSA^{+} $CD21/35^{high}$
Follicular I	$CD23^{+}$ $AA4^{-}$ $sIgM^{low}$ $sIgD^{high}$ HSA^{low} $CD62L^{+}$ $CD21/35^{int.}$
Follicular II	$CD23^{+}$ $AA4^{-/low}$ $sIgM^{high}$ $sIgD^{high}$ HSA^{low} $CD62L^{+}$ $CD21/35^{int}$
Marginal Zone	$CD23^{-}$ $AA4^{-}$ $sIgM^{high}$ $sIgD^{low}$ HSA^{+} $CD21/35^{high}$ $CD1d^{+}$

Adapted from (Cancro, 2004)

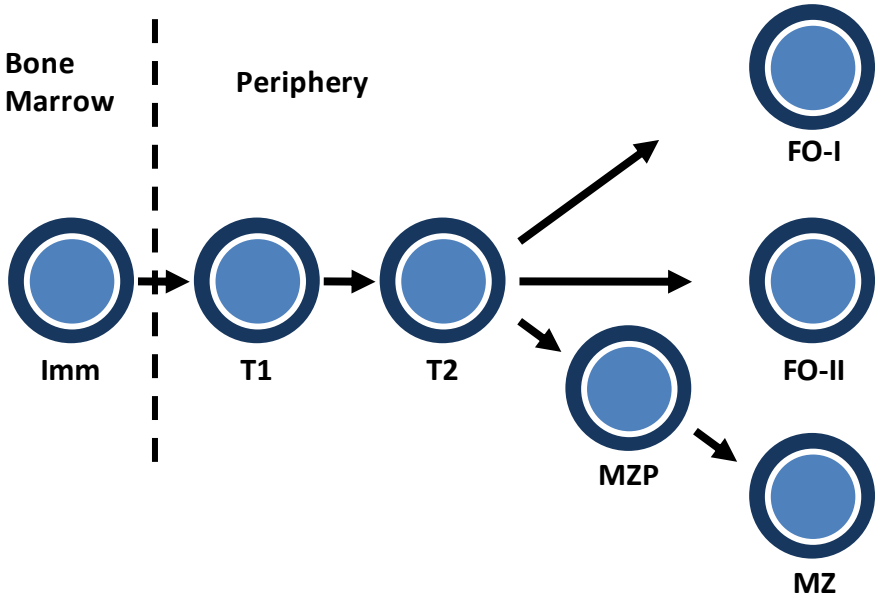

Figure 1.1.Peripheral B cell maturation from the bone marrow.
Immature B cells are generated from precursor B cells in the bone marrow, following surface expression of IgM (BCR), The immature B cells leave the bone marrow and migrate to the spleen as T1 B cells.T1 B cells develop into T2 B cells, which in turn are thought to serve as the precursor to either subsequent MZ precursor or mature FoBI and FoBII B cells. MZP cells give rise to MZ B cells. Adapted from(Allman and Pillai, 2008; Khan, 2009).

BCR in development, maintenance and survival

Maintaining the primary lymphocyte pools is a challenge for the immune system: it must balance a sufficient lymphocyte pool for ample immunological response versus the need to minimize the risk of developing an lymphocyte collection that is autoreactive and / or ineffective. The adult mouse bone marrow manufactures ~15 million immature B cells per day, yet only ~10% leave the marrow. With an estimated 95% of immature and transitional cells perishing prior to joining mature B cell pools, reflects a rigorous specificity-mediated negative and positive selection (Fulcher and Basten, 1994)(Hayakawa et al., 1999)(Wang and Clarke, 2004). Mounting evidence indicates that BCR signaling plays a key positive role operating across the entire developmental window from the bone marrow through the mature B cell stage.

Results representing that the tendency for maturation and survival depend on BCR signaling first came from experiments in which mixed-marrow chimeras were constructed from normal and xid donors (Sprent and Bruce, 1984). Further evidence includes gene targeting experiments in which components of the BCR were inactivated by germline mutations (Rajewsky, 1996) or Igβ was downregulated at the immature B cell stage (Meffre and Nussenzweig, 2002).

A fundamental change occurs in the functional consequence of BCR crosslinking following immature B cell exit toward the periphery. Newly arrived

immature B cells in the spleen, similar to bone marrow counterparts, die in response to BCR crosslinking, allowing for removal of potentially self-reactive species. Negative selection of self-reactive immature B cells operates at the T1 stage and can occur by multiple mechanisms, including deletion, anergy, or receptor editing upon Ag encounter (Cyster et al., 1996; Gay et al., 1993;Melamed et al., 1998). However, during transition from immature towards mature B cells, the response to BCR-crosslinking shifts from cell death to cell proliferation (Kaileh and Sen, 2012). *In vivo*, positive BCR signals are proposed to drive the transition into a T2 B cell due to weak BCR engagements or in a ligand-independent fashion through tonic BCR signaling (Bannish et al., 2001; Cancro and Kearney, 2004; Gu et al., 1991). Stage-specific quantitative and qualitative alterations in BCR signaling in T1 cells may protect cell clones from undergoing apoptosis due to a gradual increase in expression of intracellular signaling proteins (Bannish et al., 2001; Casola et al., 2004). Upon differentiation, T2 cells display enhanced expression of CD19, CD21. In addition, greater BR3 (BAFF-R) levels in T2 B cells allows for improved responsiveness to the BAFF survival cytokine (Rowland et al., 2010; Smith and Cancro, 2003).

Finally, BCR signaling is not only required for development of immature B cell into long-lived peripheral B cells, but also requisite for continued maintenance of the lymphocyte pool. Mature B cells undergo apoptosis upon *in vivo* BCR ablation or mutation of one of its signaling units, the Igα

polypeptide chain, and disappear from the body with a half-life of 3–6 days. Thus indicating that the BCR on resting, mature B cells transmits “tonic” survival signals into the cells, either upon interaction with ligands in the environment or spontaneously (Kraus et al., 2004; Lam et al., 1997).

BAFF mediated survival and maintenance

BAFF is the most critical soluble factor for peripheral B cell maturation and survival (Moore et al., 1999; Schneider et al., 1999). BAFF, its receptors, and its related cytokine APRIL (a proliferation inducing ligand), are members of the tumor necrosis factor (TNF) family (Schneider et al., 1999; Yu et al., 2000). Due to its near-simultaneous discovery by several laboratories, BAFF appears in the literature under multiple names: B-lymphocyte stimulator (BLyS) (Moore et al., 1999), TNF and ApoL-related leukocyte-expressed ligand-1 (TALL-1) (Shu et al., 1999), TNF homolog-activating apoptosis nuclear factor-kB c-Jun NH2-terminal kinase (THANK) (Mukhopadhyay et al., 1999). BAFF is expressed by monocytes, macrophages, dendritic cells, and neutrophils, but not by B cells themselves (Moore et al., 1999)(Kawasaki et al., 2002)(Litinskiy et al., 2002)(Scapini et al., 2003).

It is unlikely that BAFF plays a role in early bone marrow B cell development, as neither BAFF binding nor receptor transcripts are found within B cell progenitors prior to the immature B cell stage (Hsu et al., 2002). Further, populations of these early differentiative stages are altered in BAFF transgenics, knockouts, or receptor knockouts (Lentz et al., 1996)(Schiemann et al., 2001)(Mackay et al., 1999). This is in stark contrast to BAFF effect out in the periphery, as targeted deletion of BAFF ligand or BAFF-R results in a partial block at the T1 → T2 transition, leading to severe deficiency of mature B cells (Harless et al., 2001; Schiemann et al., 2001). Conversely, if BAFF

levels are experimentally elevated, the rigidity of transitional selection is "relaxed," and cells that would normally be selected against, instead survive to join the FO or MZ pool (Stohl, 2005; Stohl et al., 2005). Numbers of cells in each of these subsets are correspondingly increased (Hsu et al., 2002).

BAFF has been characterized chiefly as a pro-survival factor. Mature B cells strongly upregulated anti-apoptotic members of the Bcl-2 family, Bcl-xl and A1 in response to BAFF, whereas the upregulation of these genes in bulk transitional populations was low (Hsu et al., 2002). Conversely, an increase in pro-apoptotic Bcl-2 family members has been reported in BAFF receptor mutants (Clise-Dwyer et al., 2001). Enforced Bcl-2 expression rescues the transitional B cell block, leading to the generation of follicular B cells (Rahman and Manser, 2004; Sasaki et al., 2004), further substantiating the pattern of BAFF or BAFF-R deficiency imposes a block at the T1 → T2 maturation stage due to failed survival.

BAFF signaling contributes to BCR-mediated positive selection at the transitional developmental checkpoint, and functions in homeostasis of the mature naïve compartment. BAFF is a limiting survival factor, and only those cells that successfully compete for it remain in the FO or MZ B cell pools. The boundary of B cells reaches several million (MZ) or tens of millions (FO) in the adult mouse. These cells are quiescent and reside in the compartment for the duration of their lifespan (measured in months) unless they respond to antigen. Restrictions on BAFF levels yield a mature naïve compartment of

sufficient steady-state size and diversity for effective immune surveillance, along with sufficient quality to avoid autoreactivity (Stohl, 2005; Stohl et al., 2005).

Both BCR and BAFF-R utilizes the NFκB transcription factor family, and is essential for the development, survival and maintenance of B cells in the spleen. The inducing kinases and NFκB effectors however, are different for each receptor. As such, a brief overview of this signaling pathway is necessary.

NFκB Signaling System

Nuclear factor κB (NFκB), first identified in B cells as a transcription factor that binds to the intronic enhancer of the kappa light chain gene in B cells (Sen and Baltimore, 1986). Over the years since its discovery, members of this transcription family have been shown to play a critical role in the survival of developing B cells in the spleen, and for the antigen dependent activation and proliferation of lymphocytes. In addition, it has implicated in a wide range of biological processes, including survival, stress responses, immune responses, and maturation of various cell types. Misregulation of NFκB activity is frequently observed in various diseases including chronic inflammation and cancer, rendering the understanding of NFκB regulation critical for maintaining human health.

The NFκB family comprises dimers of related subunits that control transcription by binding κB elements in the regulatory regions of target genes (Hayden and Ghosh, 2008). The five subunits p50, p52, p65 (RelA), cRel, and RelB, encoded by NFKB1, NFKB2, RELA, REL, and RELB, respectively, share a conserved N-terminal domain (Rel homology domain) that is indispensable for DNA binding, dimerization and nuclear import (Hayden and Ghosh, 2008). The five monomers form 15 potential dimers. Generation of all monomers is transcriptionally regulated. However, the p50 and p52 subunits are synthesized from their precursor proteins p105 and p100, respectively (Bours et al., 1990; Ghosh et al., 1990; Kieran et al., 1990; Schmid et al.,

1991). Thus, different NFκB dimeric complexes are expressed cell type- and stimulus-specifically; some of the physiologically important dimers are RelA:p50, cRel:p50 and RelB:p52 (Shih et al., 2011)(Figure 1.2).

Inhibitor of NFκB protein (IκB) is a physiological regulator of the activities of the NFκB transcription factors. IκB proteins regulate NFκB activity in at least two ways: IκB binds and sequesters NFκB dimer and prevents DNA binding and transcriptional activation; it can also inhibit NFκB binding to its consensus κB DNA. The central region of IκB proteins contains multiple ankyrin repeats which folds into a single domain referred to as ankyrin repeat domain (ARD)(Baldwin, 1996; Ghosh et al., 1998). The ARD of IκBs and the RHR of NFκB dimers are primarily responsible for the IκB:NFκB complex formation (Huxford et al., 1998; Jacobs and Harrison, 1998).

The classical inhibitor proteins in the NFκB signaling system consist of the single polypeptide IκBs: IκBα, IκBβ and IκBε. Stimulus-responsive activation of the IκB kinase (IKK) results in the degradation of the IκBs to release and activate NFκB. Synthesis of IκBs may be dependent on NFκB activity, and the inducible activation of IκBs results in negative feedback. Recently, non-classical inhibitors of NFκB have been identified; p100, when present in a multimeric complex designated IκBδ, mediate NFκB inhibition in trans(Basak et al., 2007). Moreover, size exclusion chromatography analyses suggest that IκBδ activity is mediated by a ~650 kDa high molecular weight

complex, termed the IκBsome, which also contains IκBγ activity from p105 protein (Savinova et al., 2009). Finally, stimulus-responsive expression of the nfkb2 gene can provide negative feedback onto RelA:p50 dimers during prolonged TLR-mediated signaling further illustrating the pathways that which control IκBsome assembly and degradation are critical for regulating NFκB activity (Shih et al., 2009).

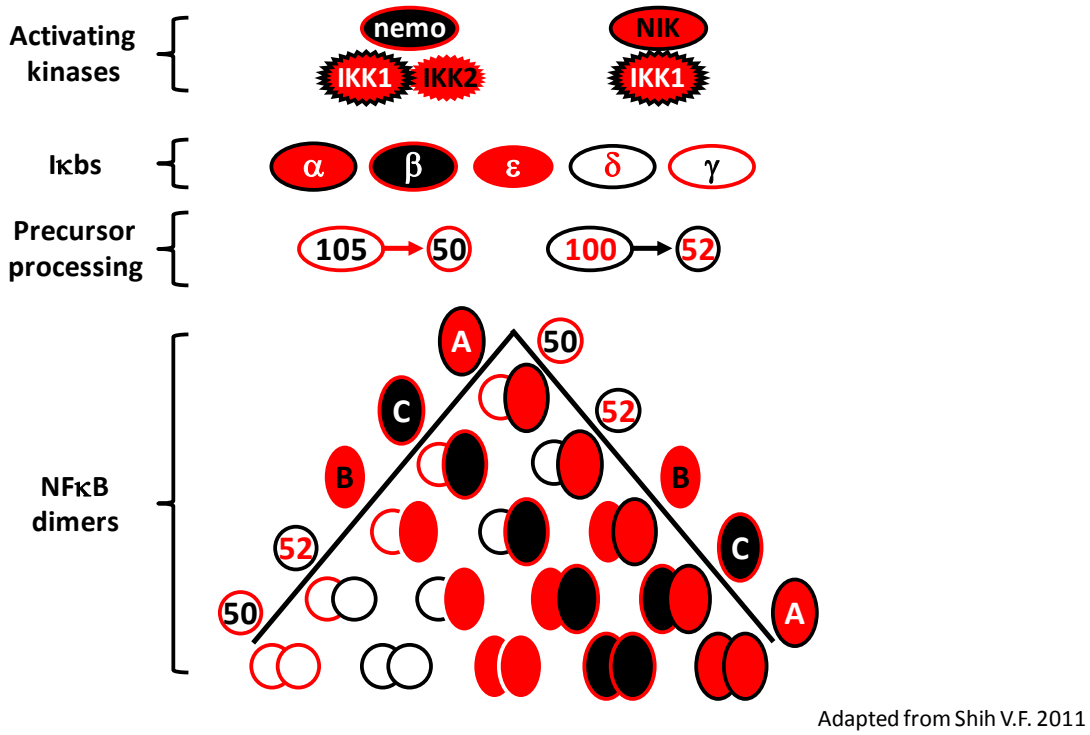

Figure 1.2.Components of the IKK-IκB-NFκB signaling system.
The IKK form canonical NEMO-containing (green) complexes and non-canonical (blue) complexes, which control the degradation of IκB proteins and precursor processing. IκBα, IκBβ, IκBε and the IκB activities within the IκBsome IκBγ and IκBδ are able to sequester NFκB dimers. The p50 and p52 NFκB proteins are generated from the processing of newly synthesized precursor proteins p105 and p100, respectively. The five NFκB family members (RelA/p65, cRel, RelB, p52 and p50) can potentially form 15 heterodimers and homodimers that can bind to a large number of κB sites in DNA. Figure adapted from (Basak et al., 2012; Shih et al., 2011).

BCR signaling activates the canonical arm of NFκB

NFκB signaling occurs through either the classical (also known as canonical) or alternative (also known as noncanonical, nonclassical) pathway (Bonizzi and Karin, 2004; Ghosh and Karin, 2002). The classical pathway involves an IκB kinase (IKK) complex encompassing three known subunits: two protein kinases (IKK1 and IKK2) and a structural/regulatory subunit (called NFκB essential modulator [NEMO] or IKKγ, in an unknown stoichiometry and NFκB activation occurs through IκB degradation (Li and Verma, 2002). The alternative pathway depends on IKK1 and NFκB-inducing kinase (NIK) and is based on regulated processing of the p100 precursor protein (Senftleben et al., 2001; Xiao et al., 2001).

BCR signaling utilizes the classical (NEMO-dependent) arm of NFκB. The stimulation of B cells via the BCR causes activation of Syk, Btk and Src family protein kinases such as Lyn, Fyn and Blk. This translocation puts Btk in close proximity to Syk, resulting in its subsequent phosphorylation. Btk, BLNK and Syk then cooperate to activate PLCγ2 and induce IP3 production and sustained Ca2+ flux. These second messengers activate PKCβ. Engagement of the BCR initiates the recruitment of PKCβ into lipid rafts. While the CBM complex, composed of Carma1/Bcl10/MALT1 has clearly been implicated in NFκB activation in B cells, but it has not been determined whether it connects PKCβ to the IKK complex (Schulze-Luehrmann and Ghosh, 2006; Weil and Israel, 2004). Following activation of NEMO-IKK1/2 complex, it phosphorylates

classical IκB molecules α ,β ,ε; leading to their degradation through the ubiquitin-proteasome pathway. This results in the release of RelA and cRel containing dimers, which then translocate to the nucleus and activate their target genes (Figure 1.3).

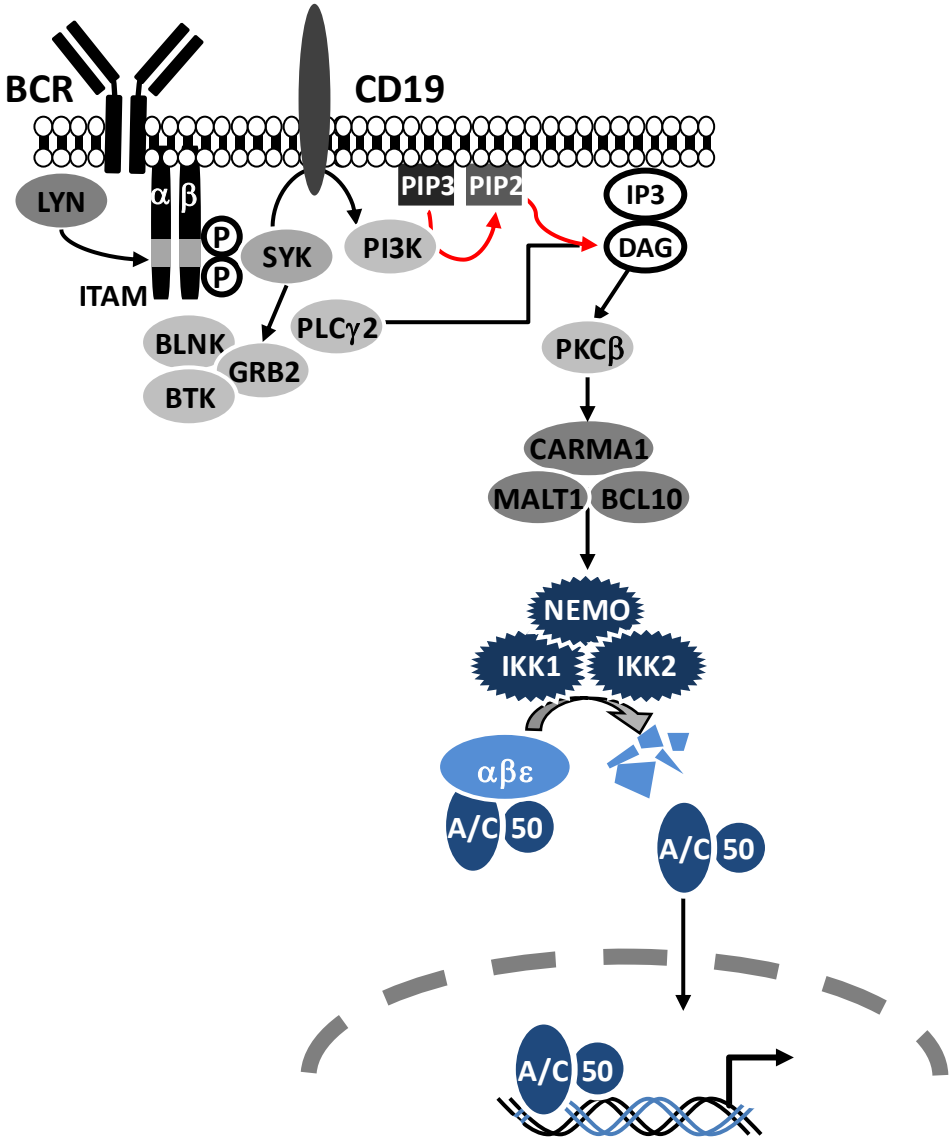

Figure 1.3. BCR activation induces NFκB in a NEMO-dependent manner.

Non-canonical NFκB activation via BAFF signaling

In contrast to BCR, BAFF-R signaling activates the non-canonical NFκB system, or NIK-dependent manner. The serine/threonine kinase NFκB-inducing kinase, NIK is an unstable protein which undergoes constitutive degradation in unstimulated cells. This process involves (TNF receptor associated factor) TRAF2 and TRAF3, as B cell lines that lack TRAF3 show greatly elevated levels of NIK and NFκB2 processing (He et al., 2006). A similar phenotype is seen in B cells that lack TRAF2 (Grech et al., 2004), suggesting that TRAF2 and TRAF3 perform co-operative but non-redundant roles in controlling the alternative NFκB pathway. Vallabhapurapu and colleagues further elucidated this pathway; BAFF-R engagement recruits the NIK/ TRAF2/TRAF3/cIAP1/2 complex to the receptor, inducing the ability of TRAF2 to ubiquitylate cIAP1/2, then redirects cIAP1/2 to ubiquitylate TRAF3, leading to TRAF3 degradation and allowing newly formed NIK to persist and activate IKK1.

NIK is essential for the induction of p100 processing, as first revealed using the Aly mice (Xiao et al., 2001). NIK / IKK1 complex then phosphorylates the serine residues at p100 C-terminus which leads to the processing of p100 and the generation of p52 through a yet unknown mechanism. p52 heterodimerize with RelB and regulate the target gene expression (Sun, 2011)(Figure 1.4). In accordance with its key role in this pathway, NFkb2 (p100 / p52) deficient B cells are non-responsive to BAFF-dependent survival

signals *in vitro* . However, the presence of mature follicular B cells in Nfkb2- and RelB-deficient mice pose an conundrum in their function of BAFF-mediated development (Claudio et al., 2002; Weih et al., 2001). We shall now take a close examination of the functional role of NFκB in B cells development and homeostasis.

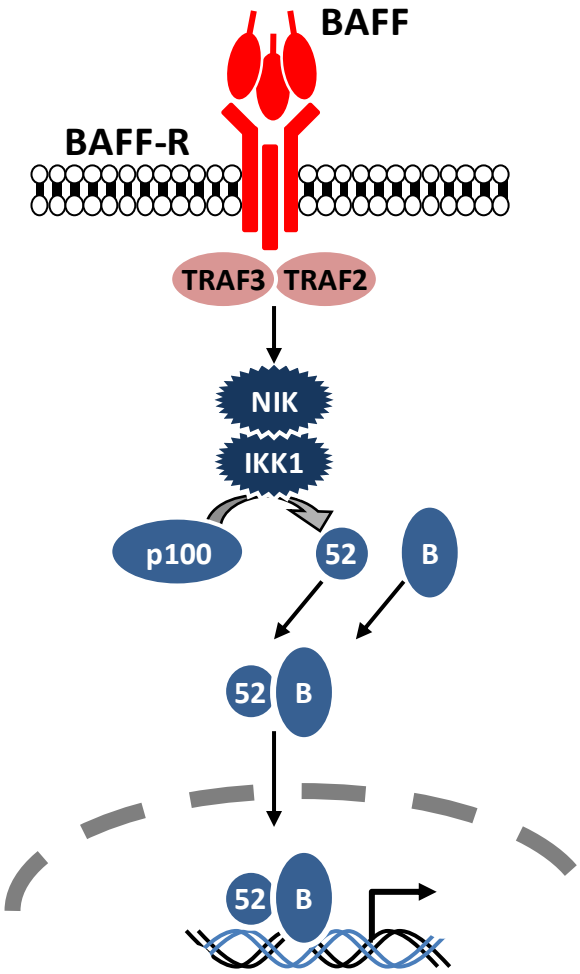

Figure 1.4. BAFF activation utilizes the non-canonical NFκB pathway.

NFκB in B cells development

NFκB proteins were identified based on binding to a highly conserved sequence referred to as the κB site within the κlight chain gene enhancer (Sen and Baltimore, 1986). Despite the importance of BCR rearrangement in the initial stages of B cell development, there is little genetic evidence which supports NFκB playing any significant role for in the B cell development within the bone marrow. None of the single or compound mutations of NFκB proteins result in a discernible block in BM B cell differentiation (Table 1.3)(Gerondakis and Siebenlist, 2010; Vallabhapurapu et al., 2008). Neither does conditional deletion of the IκB kinases affect the populations of pro- and pre-B cells (Derudder et al., 2009; Kaisho et al., 2001;Pasparakis et al., 2002).

NFκB DNA binding activity can be detected in immature B cells, particularly those that are engaged in receptor editing (Cadera et al., 2009; Verkoczy et al., 2005). Conditional deletion of NEMO, the double deletion of IKK1 and IKK2, or deletion of TRAF6 (Derudder et al., 2009) results in a lower frequency of immature B cells. In addition, double deletion of Nfkb1 and Nfkb2 genes leads to reduced numbers of immature B cells (Claudio et al., 2009). Thus illustrating the requirement of intact classical and non-classical NFκB pathways for proper immature B cell development.

Once the immature B cells leave the marrow for the periphery that a critical role in development for NFκB proteins emerges. Several genetic mutations demonstrate this point. First, cRel/RelA double-mutant fetal liver

cells produce only T1 cells following transfer to irradiated hosts (Grossmann et al., 1999; Grossmann et al., 2000). This phenotype can be partially rescued by Bcl2 transgene, while differentiation remains incomplete indicating that Rel proteins are required for differentiation as well as cell survival. Consistent with the necessity of cRel and RelA are mandatory for transition to T2 cells, conditional deletion of either NEMO or the compound deletion of IKK1 and IKK2, also leads to a blockage in T2 differentiation (Derudder et al., 2009). In sum, this suggests that signal-induced activation of classical NFκB pathway proteins is essential for T2 differentiation. The fact that single deletion of either Rel or RelA, or either IKK gene, does not significantly impair differentiation likely reflects compensatory activity of the remaining proteins.

While direct evidence has yet to revealed, the most likely source of NFκB-induction in T1 cells is the BCR. This is most clearly exemplified by conditional deletion of CD79a, the BCR signal transducing module, in immature B cells in vitro (Schram et al., 2008; Tze et al., 2005). Absence of this protein leads to dramatic alteration of the gene expression profile in these cells towards a pattern that is more similar to that seen in pro-B cells. Additionally, deficiency of several cytoplasmic signaling molecules, such as Btk, PLCy, and BCAP, which transduce BCR signals in mature cells, also affect peripheral differentiation at transitional stages (Allman et al., 2001; Cancro et al., 2001; Doody et al., 2001; Fruman et al., 1999). Thus,

constitutive BCR signaling maintains the state of T1 cells and is required for developmental progression.

NFκB proteins have essential roles in the generation and/or maintenance of mature B cells. Of the two major subsets of B cells produced in the adult, MZ B cells are more susceptible to NFκB deficiency. Several Rel-family single gene deficiencies, including Nfkb1 and RelB show a reduction in MZ B cells (Grumont et al., 1998; Weih et al., 2001). Follicular (FO) mature B cells are less affected by single gene deficiencies, though they are significantly reduced in several compound deficiencies such as Nfkb1/2, Nfkb1/RelB, and RelA/Rel (Claudio et al., 2002; Grossmann et al., 2000;Weih et al., 1997). Both kinds of mature B cell subsets are also reduced in mice that lack IKK1 and IKK2 or NEMO (Gerondakis et al., 2006).

These observations demonstrate that both canonical and noncanonical pathway NFκB proteins are required for mature B cell generation and/or maintenance, and that their function must be induced via the IKKs. Together, these findings indicate that, analogous to studies directly investigating the BCR or BAFF-R, a combination of both NFκB pathways is necessary for the full function of either receptor (Caamano et al., 1998; Claudio et al., 2002; Grumont and Gerondakis, 1994; Grumont et al., 1998)

Table 1.3.Effects of NFκB deletions in B cell development.

Genotype	Bone Marrow	Periphery				References
	Imm.	T1	T2	FO	MZ	
wild type	+++	+++	+++	+++	+++	
***relb*$^{-/-}$**	+++	+++	+++	+++		Weih 1995 Weih 2001
***crel*$^{-/-}$**	+++	+++	+++	+++	+++	Kontgen 1995
***rela*$^{-/-}$*tnf*$^{-/-}$**	+++	+++	+++	+++	+++	Alcamo 2001
***nfkb1*$^{-/-}$**	+++	+++	+++	+++	++	Sha 1995
***nfkb2*$^{-/-}$**	+++	+++	+++	++	+	Caamaño 1998 Franzoso 1998
***ikk1*$^{-/-}$**	+++	+++	++	+	+	Kaisho 2001 Senftleben 2001
***ikk2*$^{-/-}$**	+++	+++	++	+	+	Pasparakis 2002
***nemo*$^{-/-}$**	+++	++	+	+	+	Pasparakis 2002 Sasaki 2006
***rela*$^{-/-}$*crel*$^{-/-}$**	+++	++	+			Grossmann 2002
***nfkb1*$^{-/-}$*nfkb2*$^{-/-}$**	++	+				Franzoso 1997 Claudio 2002
***ikk1*$^{-/-}$*ikk2*$^{-/-}$**	++	+				Derudder 2009

NFκB signaling enables crosstalk between BCR and BAFF-R

B cells that fail to signal through either the BCR or BAFF-R die, indicating that both receptors are necessary but neither is sufficient for B cell survival. Conditional deletion of the BCR or its immediate downstream signaling components leads to the death of most B cells despite intact BAFF signaling (Kraus et al., 2004; Lam et al., 1997). Analogous to this, mice with mutations in the gene encoding BAFF-R or a deficiency of BAFF have a dramatic reduction in B cell numbers, regardless of a proper BCR repertoire (Lentz et al., 1998). Regulation of immune responses through multiple phases is a dynamic process, as such controlling B cell homeostasis via two receptors provides flexibility in modulating differential survival requirements. Thus making BCR - BAFF-R crosstalk essential (Kaileh and Sen, 2012).

For years, numerous reports have examined the mechanisms which enable BAFF-R and BCR crosstalk. As such multiple models have been proposed. The functional role of BAFF-R and BCR crosstalk in B cell homeostasis has been the topic of several recent reviews (Cancro, 2009; Khan, 2009;Mackay et al., 2010). An encompassing summary of all the proposed models is beyond the scope of this chapter. However, following examination of the current literature, an overarching observation becomes apparent; Canonical and non-canonical NFκB transcription pathway is critical in the integration of BAFF-R and BCR crosstalk. Prior to examination of the prevailing NFκB-mediated mechanisms of this crosstalk, it is prudent to

mention that PI3K- and MAPK-dependent pathways exist, are non-exclusive, and have merit.

As mentioned previously, BAFF-R signaling mainly activates the noncanonical NFκB pathway, which involves IKK1-mediated phosphorylation and the subsequent proteolytic processing of p100 to p52, which preferentially dimerizes with RelB, and p52:RelB translocate to the nucleus (Claudio et al., 2002; Senftleben et al., 2001). Because proteolysis is irreversible, processing to p52 leads to the elimination of the precursor protein p100. In the absence of other inputs, BAFF-R signaling rapidly depletes the available p100 pools (Stadanlick et al., 2008). However, BCR tonic and induced signaling produces de novo synthesis of p100 (Castro et al., 2009; Stadanlick et al., 2008). These results suggest that BCR signaling through the classical NFκB pathway provides BAFF with a p100 supply for sustained activation of the alternative NFκB pathway, and the subsequent expression of antiapoptotic genes to allow continued B cell survival (Castro et al., 2009).

A simple model for the co-receptor crosstalk would be that the BAFF-R and the BCR share identical signaling components. Recently, it was shown that BAFF-R uses the BCR complex to promote follicular B cell survival in a SYK-dependent manner (Schweighoffer et al., 2013). This idea was previously demonstrated by Shinners et al, as Btk (Bruton's tyrosine kinase) deficient B cells survive poorly relative to wild-type controls in *vitro* cultures containing BAFF. This is surprising given that Btk is required for B lymphocyte survival,

proliferation, and differentiation in response to BCR activation (Rawlings, 1999). In the same study, BAFF stimulation triggered classical NFκB components via degradation of Iκbα and activation of cRel containing dimers in a Btk- and phospholipase C-γ2- dependent (Hikida et al., 2003; Shinners et al., 2007). This activation of cRel is key, as it is required for the BCR-mediated expression of BAFF-R transcripts (Smith and Cancro, 2003)(Castro et al., 2009). Thus, cRel is not only important in the regulation of B cell survival directly by inducing the expression of antiapoptotic genes, such as A1 and Bcl-XL, but also indirectly via increasing the expression and function of BAFF-R (Castro et al., 2009; Stadanlick et al., 2008).Through the intermingling of signaling components like Btk and Syk, augmentation of BCR induced canonical NFκB signals by BAFF can be achieved.

These observations suggest that synergy between BCR and BAFF-R for B cell survival may ultimately lead through activation of classical NFκB, despite the critical role of the noncanonical NFκB pathway in mediating BAFF-dependent survival signals. Consistent with these ideas; expression of constitutively active IKK2 (Ikk2ca), which induces canonical NFκB, in mice completely restores the B cell deficiency in BAFF-R-deficient mice (Sasaki et al., 2006). This includes generation of follicular mature and marginal zone B cells and restoration of splenic architecture. B cells from Ikk2ca mice have higher nuclear levels of classical NFκB proteins, suggesting that generation and survival of mature B cells in these animals is entirely dependent on

canonical NFκB pathway signaling (Kaileh and Sen, 2012; Sasaki et al., 2006).

Focus of our study

NFκB was initially discovered in B cells. Despite decades of research in B-lymphocytes, the exact functions of canonical and non-canonical NFκB in B cells remain an area of great debate. In addition, crosstalk and interdependent regulation between the two pathways enhances the complexity which confounds many researchers in this field. My thesis will attempt to clear some of this confusion by performing a quantitative examination of NFκB signaling to determine the functions of canonical and non-canonical NFκB in B cell survival and development. My specific aims are designed to answer the following issues:

From the introduction we have discussed evidence which shows that the BAFF-R is capable of activating canonical and non-canonical NFκB. It was also noted that both of these signaling pathways are critical in the development of peripheral B cells. Despite the extensive research in BAFF signaling, the exact role of canonical NFκB in BAFF-mediated survival and maturation. In chapter two, we will attempt to elucidate this function.

The previous section discussed the mechanisms by which BCR and BAFF crosstalk is enabled by NFκB. But these are primarily homeostatic models. BAFF has been shown to prime, or prepare B cells for introduction of foreign antigen(Patke et al., 2006). Indeed, co-stimulation of BAFF-R and BCR will lead to enhanced B cell expansion *in vitro*. In chapter three we shall try to

uncover the mechanisms by which BCR and BAFF-R signals synergize in the context of B cell activation.

CHAPTER 2:

B cell survival and development is dependent on the coordination of NFκB family members RelB and cRel.

Introduction

B cell development originates in the bone marrow, where hematopoietic stem cell precursors which commit to the B cell lineage will initiate immunoglobulin heavy-chain gene rearrangements. If rearrangement is successful, they will alter their light chain loci and differentiate into immature B cells. Cells which generate functional B cell antigen receptors (BCR) eventually leave the bone marrow and migrate to the spleen to complete their maturation process. The first B cells to arrive are referred to as transitional-1 B cells (T1). T1 B cells are still subject to negatively selective pressures; strong antigenic signals lead to apoptosis. In later transitional stages, some of the immature B cells (T2) have built a tolerance to antigen-receptor signaling, and 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 (Gerondakis and Siebenlist, 2010)(Pillai and Cariappa, 2009).

As mentioned above, B cell Activation Factor receptor (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 (Harless Smith and Cancro, 2003)(Schiemann et al., 2001). BAFF initiates the non-canonical NFκB pathways 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 (Claudio et al., 2002). It has also

been reported that BAFF can utilize the canonical NFκB pathway (Shinners et al., 2007)(Stadanlick et al., 2008). Gene-targeted deletions of NFkB1 (p50), the primary binding partner of RelA and cRel, result in defective survival of B cells in response to BAFF (Hatada et al., 2003). Also, B cell maturation phenotype similar to that of BAFF / BAFF-R deficient mice is only observed in mice lacking components of both classical and alternative pathways (*nfkb1*$^{-/-}$ *nfkb2*$^{-/-}$*) (Claudio et al., 2002)*.

The primary function of BAFF is survival, which is believed to be controlled mainly by non-canonical NFκB (Cancro, 2009). However, antigenic activation of canonical cRel dimers can provide strong survival signals through upregulation of anti-apoptotic bcl-2 family proteins (Owyang et al., 2001)(Feng et al., 2004). In addition, BAFF has been shown to control other critical B cell functions such as metabolic fitness through a PKCβ- and Akt-dependent mechanism(Patke et al., 2006). From these observations we question what is the primary driver of BAFF mediated survival; NIK mediated, non-canonical NFκb , driven by Relb containing dimers or is the canonical arm of NFκB elicited by cRel (or RelA) containing dimers? Or perhaps this function is controlled by an NFκB-independent pathway (Figure 2.1). In addition, we question if there are other distinct B cell functions controlled by NFκB downstream of BAFF signaling?

In this chapter I will delineate the distinct and overlapping roles of canonical and non-canonical NFκB signals in BAFF B cell biology. From our

studies we find that, in addition to RelB, BAFF-R stimulation does indeed activate cRel containing dimers in a dose dependent manner. And while BAFF mediated survival signals are primarily controlled by RelB, cRel is also important for *in vitro* survival.

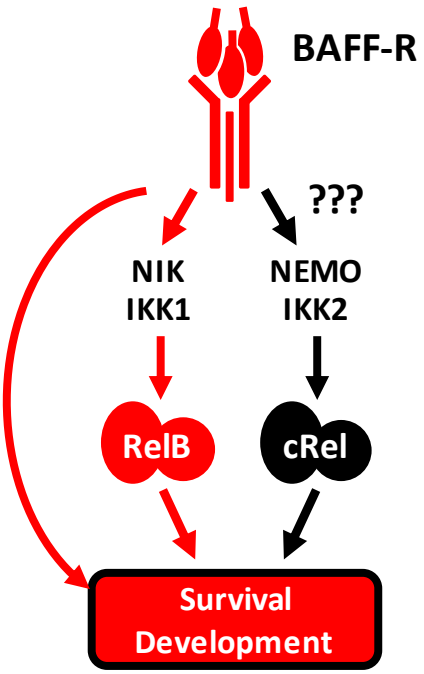

Figure 2.1. Coordination of canonical and non-canonical NFκB signaling system in BAFF-mediated survival and development.
BAFF-R is a key regulator of peripheral B cell maintenance. These signaling functions can occur in an NFκB-independent manner (left-most red arrow), as well as a NIK-dependent, non-canonical NFκB manner; mediated through RelB activity (red arrow). However, a third unexplored pathway, via NEMO-dependent canonical NFκB pathway may also play a functional role.

Results

BAFF stimulation activates canonical and non-canonical NFκB pathways.

To examine what NFκB dimers are controlling BAFF-R signaling functions, we performed an electrophoretic mobility shift assay (EMSA) on primary *wild type* B cells stimulated with 50 ng/ml of BAFF. The gel illustrates BAFF's ability to activate multiple NFκB dimers (Figure 2.2a) with three NFκB-specific bands are apparent following stimulation. These three bands were subsequently identified in supershift assays using RelA, cRel, RelB and p50 specific antibodies in gel supershift assay (data not shown). We identified the middle band, indicated by a red arrow, to be RelB-containing dimers. While the bottom band, marked with a blue arrow is p50:50 dimers. Because RelA and cRel have similar molecular weights, it is difficult to determine the kinetics of RelA and cRel using standard EMSA techniques.

A new EMSA technique pioneered in our lab by Vincent Shih utilizes multiple NFκB antibodies to examine specific activities of NFκB family members. Briefly, pre-incubating nuclear extracts with anti-RelA and anti-cRel antibodies will shift away the superfluous bands, allowing us to focus only on BAFF-mediated RelB activation. With this assay, we found that BAFF stimulation will activate continuous, non-canonical RelB containing dimers, matching previous reports (Claudio et al., 2002). In addition, we find that BAFF indeed can activate canonical NFκB pathway in the form of transient RelA

containing dimers and persistent cRel; RelA activity is very transient; peaking at one hour and back to basal levels within 8 hours. cRel is not as short lived, the peak occurs ~ 3 hours , which follows RelA (Figure 2.2). Finally, we also see that this activation occurs in a dose dependent manner, as B cells stimulated with BAFF for 24 hours have increased cRel and RelB activity with titrating levels of BAFF (Figure 2.3).

We next examined BAFF-induced activation of the alternative NFκB pathway. As expected, BAFF stimulation results in p100 processing to p52 in *wild type* B cells while the BCR induced canonical stimuli, anti-IgM does not (Figure 2.4). We then turned to BAFF activation of NEMO-dependent canonical NFκB. For this, total cellular extracts from wild type B cells stimulated with either BAFF or anti-IgM were immunoprecipitated with antibodies specific to NEMO, and the resulting immuno complexes were subjected to an *in vitro* kinase assay (Figure 2.5a). We observed increases in IKK activity in the BCR stimulated B cells, but little to none in the BAFF counterparts. We further examined the degradation of classical Iκbα under BAFF conditions, and again saw little decrease in total Iκbα levels (Figure 2.5b). From these results, it appears that that the BAFF induction of RelA and cRel dimers does not occur in the manner consistent with a typical NEMO routine.

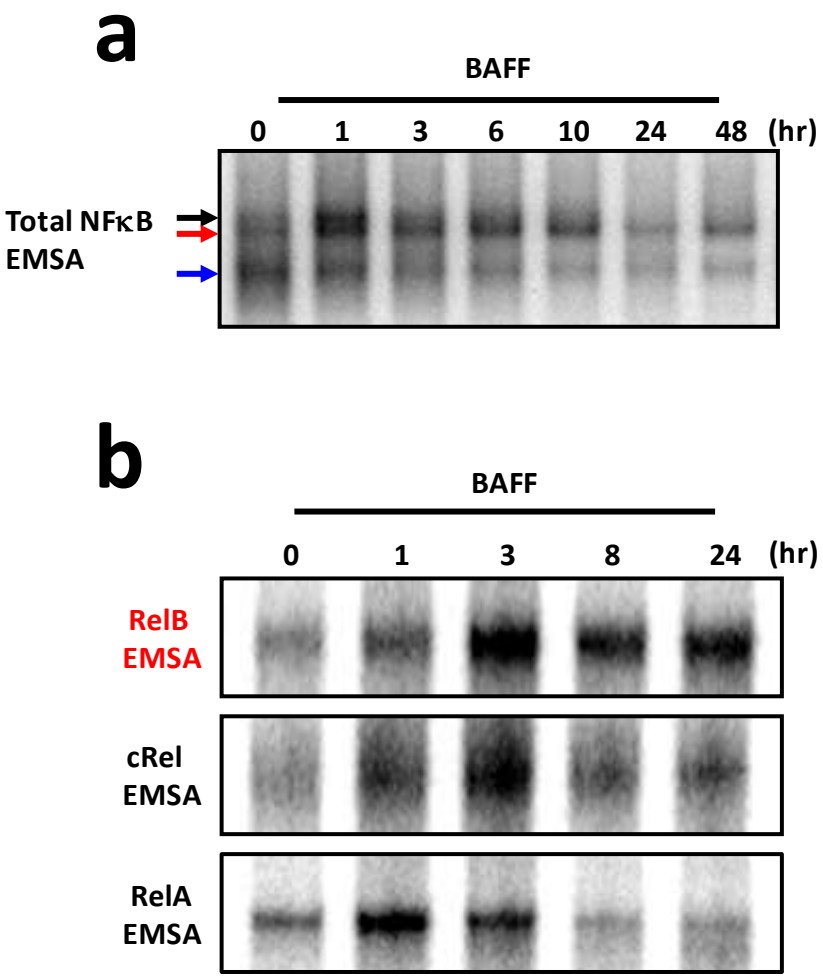

Figure 2.2.BAFF-R stimulation activates the NFκB signaling system.
(a) EMSA of *wild type* B cells stimulated with 50ng/ml of BAFF for the indicated time points.
(b) RelB EMSA and cRel EMSA of wild type B cells are utilized to reveal their respective activity kinetics following BAFF-R perturbation.

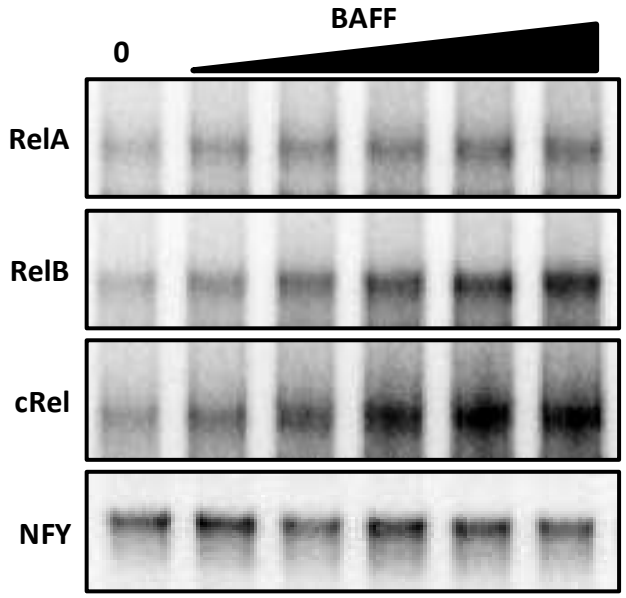

Figure 2.3. BAFF activates RelB and cRel in a dose dependent manner.
EMSA of wild type B cells stimulated with 0 to 200ng/ml of BAFF ligand for 24 hours, then analyzed for NFκB activity via EMSA. NFY is used to ensure equal loading of samples.

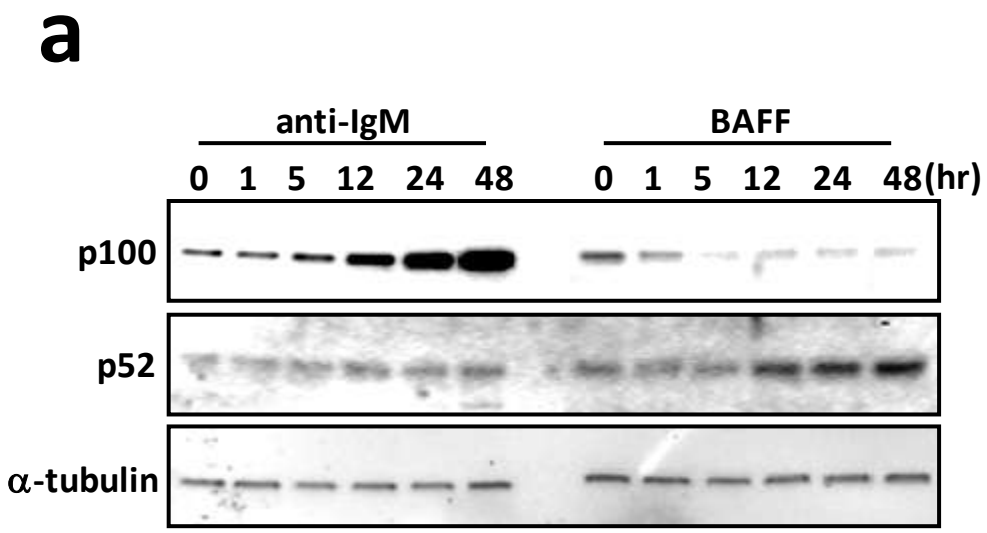

Figure 2.4. BAFF stimulation will lead to processing of p100 to p52.
(a) *Wild type* B cells are stimulated with 50ng/ml of BAFF for 48hrs in culture, and p100 and p52 levels are examined at the indicated time points by immunoblotting.

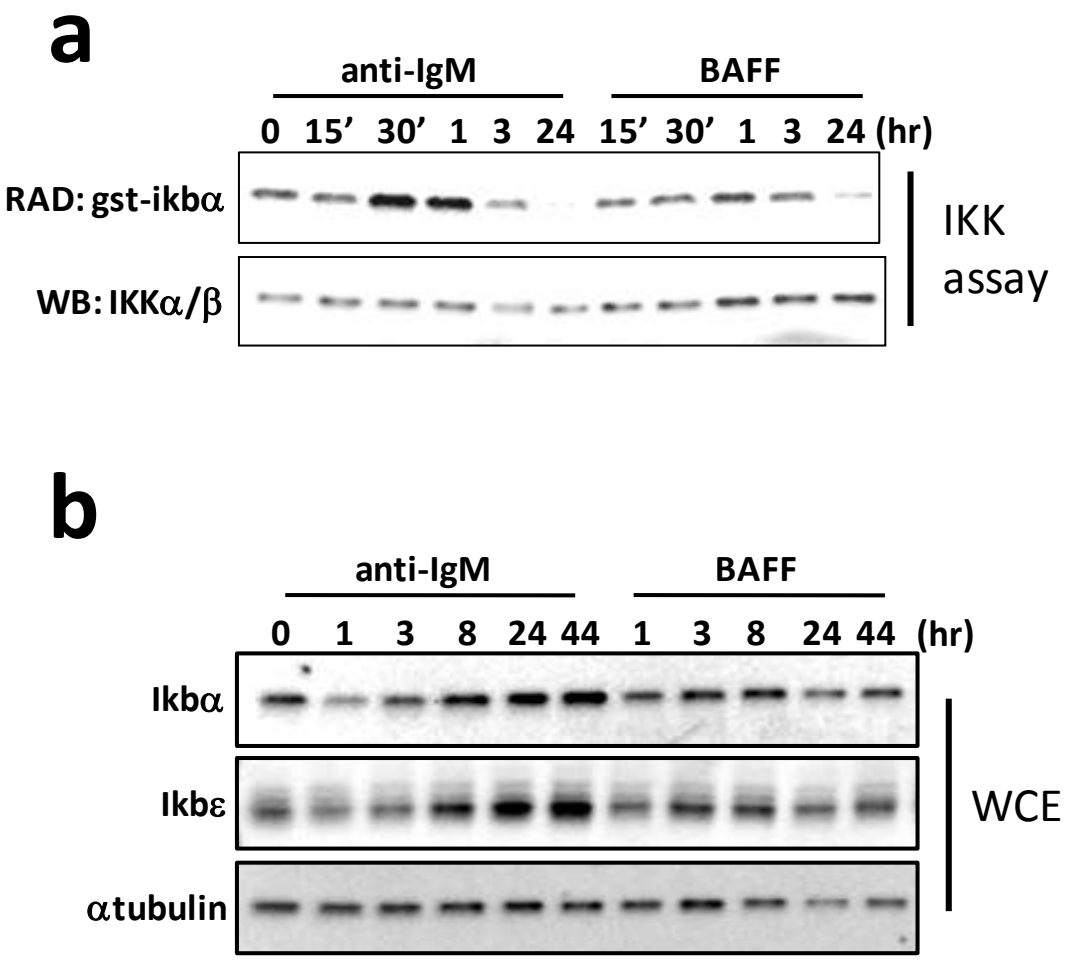

Figure 2.5. BAFF activation of RelA and cRel does not appear to occur in a NEMO-dependent manner.
(a) NEMO-associated kinase activity determined in *wild type* B cells in an *in vitro* IP-kinase assay upon stimulation with 5μg/ml of anti-IgM or 50ng/ml of BAFF.
(b) Western blot analysis of whole-cell protein levels for canonical IκBs alpha and epsilon examined in wild type B cells stimulated with anti-IgM or BAFF.

RelB and cRel are required for BAFF-mediated survival *in vitro.*

From our biochemical analysis, we have established that BAFF stimulation can activate both RelB and cRel dimers. We then decided to look at the effect of RelB and cRel deletion in BAFF survival. Whole splenic B cells were isolated from *relb*$^{-/-}$ *, crel*$^{-/-}$*,* and *relb*$^{-/-}$*crel*$^{-/-}$ deficient mice, and stimulated with BAFF for up to four days. Using an in-vitro survival assay, where cell viability was observed with fluorescent marker 7-AAD, we see that RelB deficient B cells respond weakly to the BAFF stimuli (9.3% live cells in culture compared to 33% of wild type controls) illustrating the importance of RelB in BAFF-mediated survival (Figure 2.6a, left panel). Surprisingly, we find that cRel deletion also displays less survival than BAFF stimulated wild type controls, however not to the same extent as RelB, implicating itself as a mediator of BAFF survival. We then examined the consequence of compound deletion of RelB and cRel using the *relb*$^{-/-}$*crel*$^{-/-}$ mouse These B cells proved to have the most severe phenotype, with survival being only 4.5%, two-fold less than RelB knock out B cells, and seven-fold worse than wild type controls. This observation suggests to us a compensatory functions of RelB and cRel in BAFF survival (Figure 2.6a right panel).

Attenuation of apoptosis by BAFF correlates with changes in the ratios between Bcl-2 family proteins in favor of survival, primarily by reducing the pro-apoptotic Bak and increasing its pro-survival partners A1 and Bcl-xL (Hatada et al., 2003)(Do et al., 2000). We examined expression of these two

pro-survival genes by qPCR and surprisingly we found that their expression is dependent on canonical cRel activity, but not on RelB (Figure 2.7). While this may explain the decreased survival observed in the *crel*$^{-/-}$ B cells, it does not the account for the defect in *relb*$^{-/-}$cells. For this reason we have proposed to determine the RelB-dependent survival genes using RNAseq and chromatin immunoprecipitation.

Transcription of cRel, nfkb2, and Relb (as well as nfkb1) is controlled by NFκB in B cells (Castro et al., 2009)(Grumont and Gerondakis, 1994)(Perkins, 2007). The observation that cRel knockout B cells are defective in BAFF mediated survival could be a consequence of reduced RelB expression in these cells; thus an impaired non-canonical signaling system. However, we examined the expression of key non-canonical mediators RelB or nfkb2 in cRel deficient B cells and found the expression of both these NFκB family members are similar to wild type controls (Figure 2.8). With the non-canonical signaling system intact in *crel*$^{-/-}$B cells, we can say with greater confidence that cRel is indeed required for BAFF survival *in vitro*, and double deletion of RelB and cRel results in a greater deficit in this survival phenotype.

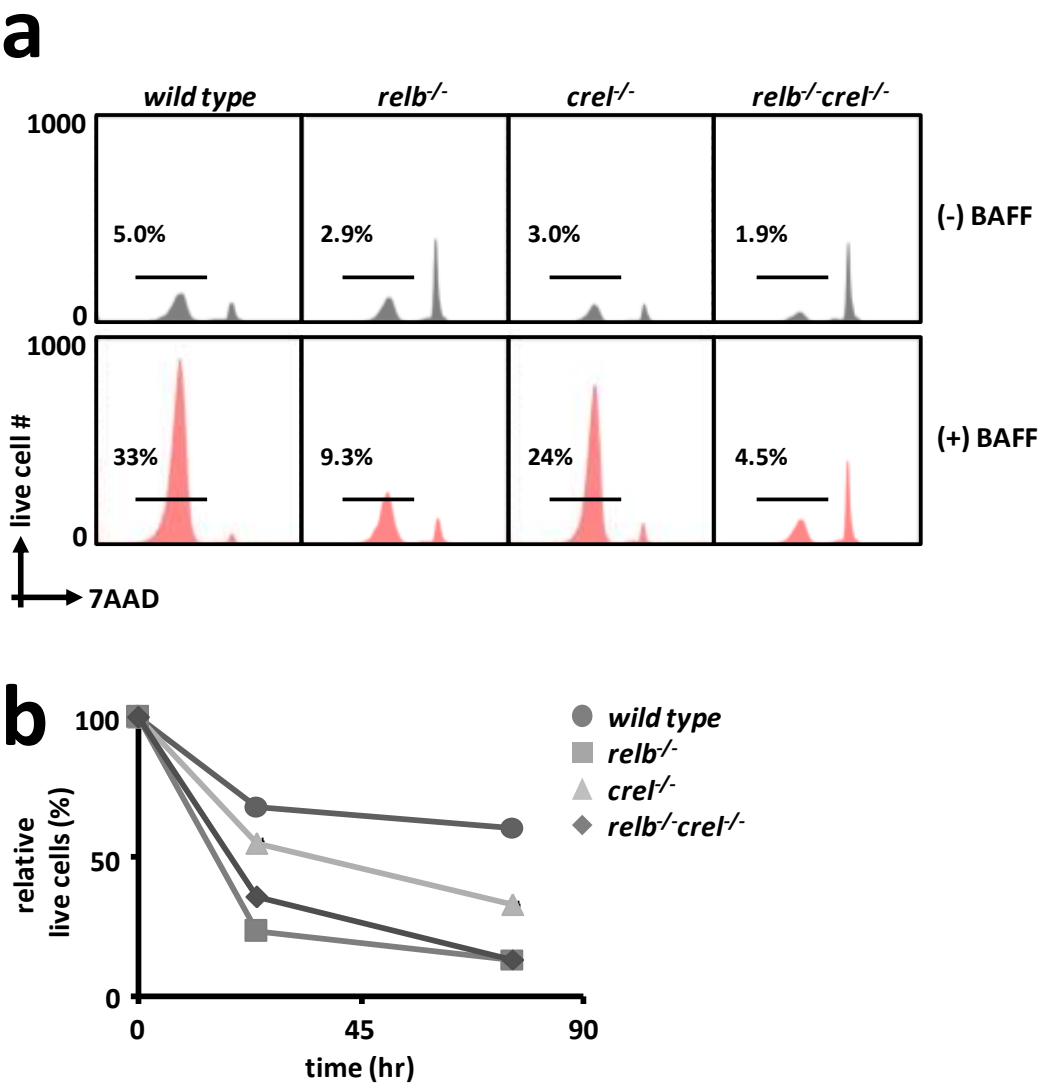

Figure 2.6. RelB, and to a lesser extent cRel, is required for BAFF-mediated survival in vitro.
(a) In vitro survival assay with 7-AAD,FACS analysis of whole splenic *wild type, relb*$^{-/-}$ *crel*$^{-/-}$*, and relb*$^{-/-}$*crel*$^{-/-}$ B cells stimulated with BAFF ligand following three days in culture. Numbers represent the percentage of live cells (7AAD^{+}) found in culture.
(b) Graphical representation of the FACS plots.

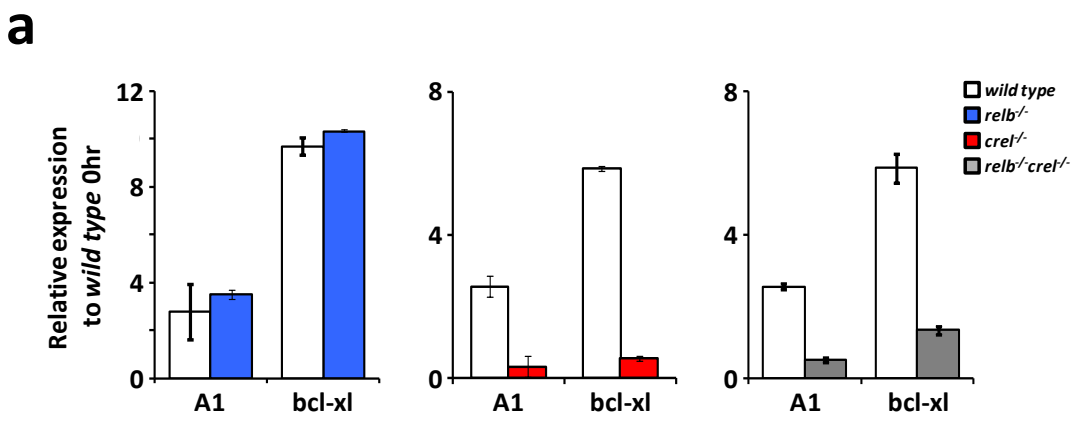

Figure 2.7. cRel is responsible for expression of pro-survival genes A1 and bcl-xl in BAFF stimulated cells.
(a) qPCR analysis of BAFF stimulated cells 3hrs after stimulation. Left panel: *wild type* vs. *relb*$^{-/-}$B cells, middle panel: *wild type* vs. *crel*$^{-/-}$, and right panel: *wild type* vs. *relb*$^{-/-}$*crel*$^{-/-}$B cells. Gene induction normalized to *wild type* unstimulated B cells.

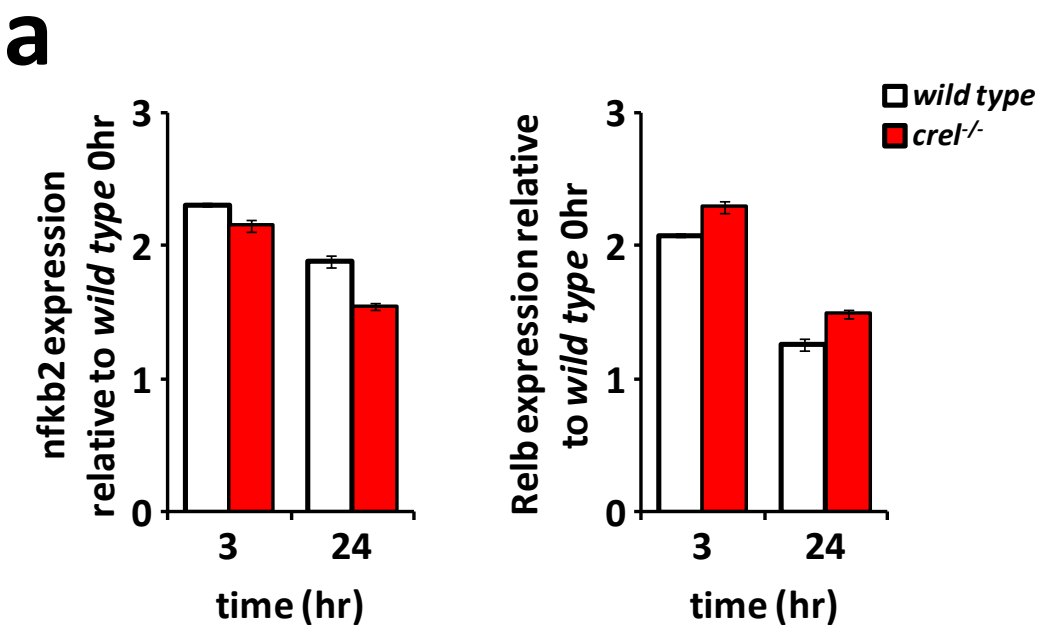

Figure 2.8. cRel deletion has little to no effect on non-canonical NFκB components relb or nfkb2.
(a)Gene expression of nfkb2 and RelB in BAFF stimulated *wild type* and cRel deficient B cells by RT-PCR. Gene induction normalized to *wild type* unstimulated B cells.

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

We have shown that RelB and cRel are vital to the survival functions of BAFF in vitro. Given the fact that BAFF also plays a substantial role in peripheral B cell development, next we examined the effects B cell development in the spleen following the loss of Relb, cRel, or both. Again, deletion of the either the BAFF ligand or the BAFF-R leads to a developmental block at the T1 stage; ensuing in complete loss of follicular and marginal zone B cells, and fewer T2 B cells (Schiemann et al., 2001). Using a FACS strategy outlined here (Allman and Pillai, 2008); we identified the peripheral B cell populations of *wild type*, *relb*$^{-/-}$, *crel*$^{-/-}$, and *relb*$^{-/-}$*crel*$^{-/-}$ mice.

Upon first examination, we see that total B cells populations (using the B cell marker B220) are unaffected when cRel is absent, however we do note a modest decrease in the *relb*$^{-/-}$spleen. This defect is more pronounces however with compound deletion of both NFκB family members (Figure 2.9). The B cells were further subdivided into mature and immature subsets using the AA4.1 surface marker. Our analysis showed that the mature B cell populations are unaffected with single deletion of RelB or cRel; the same statement holds true for the immature compartment. It is when we investigate the *relb*$^{-/-}$*crel*$^{-/-}$ spleen we can find that these ratios transpose, as there is a decrease in AA4.1^{-} mature B cells and an amplification of AA4.1^{+} immature B cells compared to the *wild type* populations (Figure 2.9).

We then focused our scan toward the mature B cell subsets; follicular B cells and marginal zone B cells. Looking at these four mouse models we note the cRel knockout in general phenocopies the wild type control. There is however, a modest reduction of marginal zone B cells, which falls in line with previous reports (Cariappa et al., 2000). FACS analysis of *relb*$^{-/-}$ spleen however has complete loss of marginal zone B cells population. This marginal zone defect also appears in the double knockout mouse, suggesting that RelB is required for proper marginal zone development, confirming previous reports (Weih et al., 2001) (Figure 2.10). Normal amounts of follicular mature B cells are present in the RelB and cRel single knockout mice compared the *wild type* mouse. The double knockout mouse however shows to have less mature follicular B cells, showing that the decrease in total mature B cells is a combination of no marginal zone B cells and fewer FO B cells (Figure 2.10).

In general the developmental faults in single knockouts mice are minimal; the *relb*$^{-/-}$*crel*$^{-/-}$animals on the other hand display a more dramatic maturation defect. FACS analyses of this animal uncover fewer total B cells, increased immature B cells, and a minority a mature B cells (Figure 2.9) in contrast to wild type. Dissection of immature B cells population reveal an increased proportion of transitional (T1) B cells, as well as an increased percentage of T2 B cells (Figure 2.11). The faults stemming from these two immature B cell progenitors would have drastic effect on the mature B cell populations; corresponding with our observations of a 33% decrease in the

follicular B cell population in relation to wild type controls (Figure 2.10).The T1 B cells are the initial class of B cells which gives rise to the mature populations (Allman et al., 2001). With the increased T1 B cells perceived in the double knockout mouse, yet not evident in the either single deleted mouse, leads us to believe that both RelB and cRel are required for proper peripheral B cell development. While the *relb*$^{-/-}$*crel*$^{-/-}$ developmental phenotype is not a severe as BAFF deficiency, the blockage at the T1 stage is the same(Harless et al., 2001). Along with our biochemical data noted from the previous results section, we propose that defective BAFF signaling may be responsible for the reduction in peripheral B cell formation.

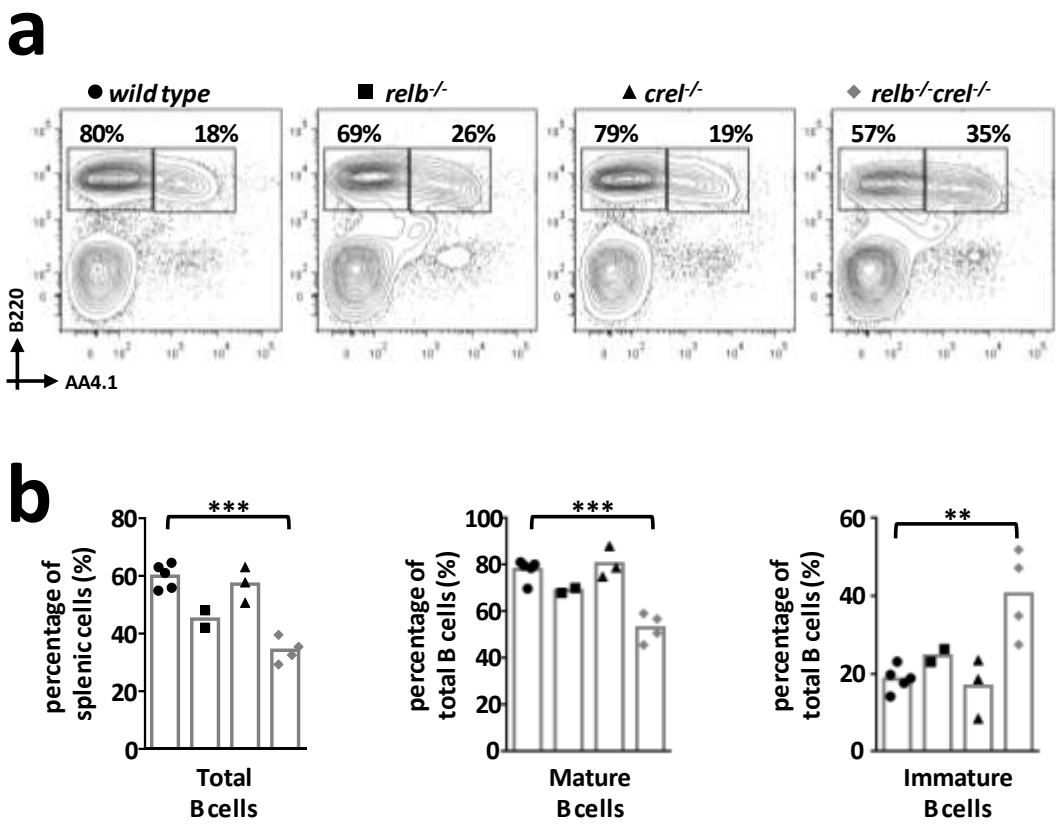

Figure 2.9. Deficiency in RelB and cRel result in fewer total B cells, more immature B cells.

(a) Fewer mature B cells and increased immature B cells are present in *relb*$^{-/-}$*crel*$^{-/-}$ spleen. Identification of mature (B220^{+}AA4.1^{-}) and immature B cells (B220^{+}AA4.1^{+}) by FACS from of whole splenic extracts from *wild type, relb*$^{-/-}$*, crel*$^{-/-}$*, and relb*$^{-/-}$*crel*$^{-/-}$ mice. (b) Scatter plots are graphical representation of FACS plots: *wild type* (●) *relb*$^{-/-}$(■) *crel*$^{-/-}$(▲) and *relb*$^{-/-}$*crel*$^{-/-}$(♦).

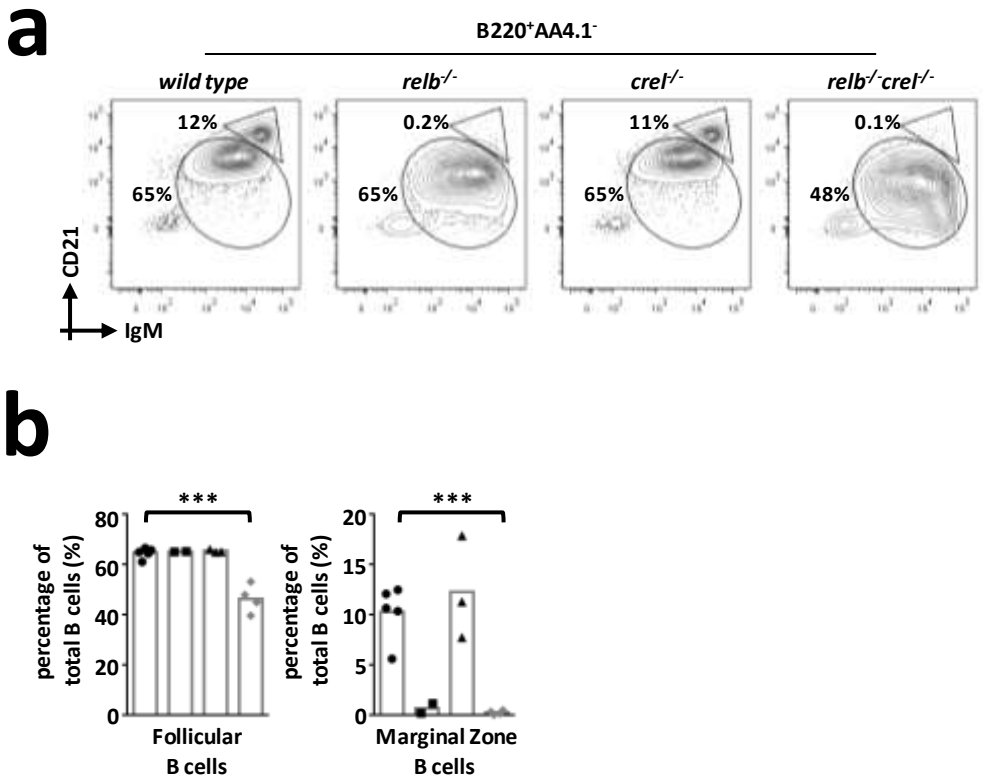

Figure 2.10. Development of mature peripheral B cells is impaired with the loss of cRel and RelB.

(a) Identification of mature B cell subsets through FACS analysis. Mature B cells ($B220^{+}AA4.1^{-}$) are subdivided into mature follicular B cells ($CD21^{+}IgM^{+}$) and mature marginal zone B cells ($CD21^{high}IgM^{+}$) by FACS from of whole splenic extracts from *wild type, relb*$^{-/-}$*, crel*$^{-/-}$*, and relb*$^{-/-}$*crel*$^{-/-}$ mice.

(b) Graphical representation of FACS plots is represented by scatter charts: *wild type* (●) *relb*$^{-/-}$(■) *crel*$^{-/-}$(▲) and *relb*$^{-/-}$*crel*$^{-/-}$(♦).

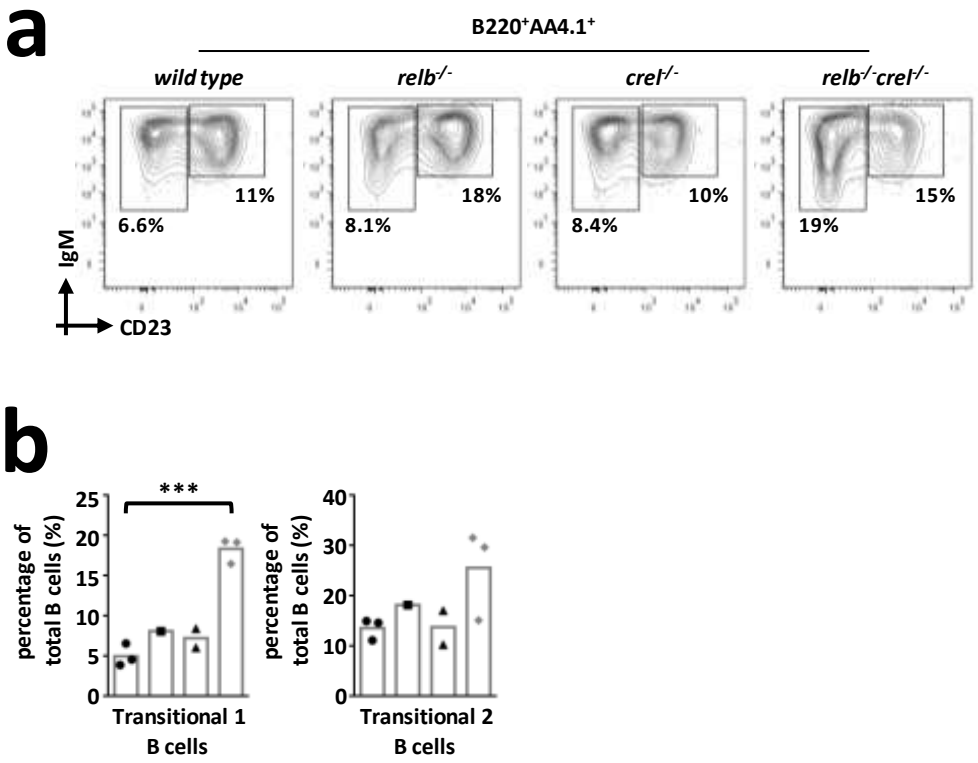

Figure 2.11. Compound deletion of RelB and cRel results in developmental block at transitional one stage.

(a) Identification of immature B cell subsets through FACS analysis. Immature B cells ($B220^+AA4.1^+$) are subdivided into transitional one (T1) B cells ($CD23^-IgM^+$) and transitional two (T2) B cells ($CD23^+IgM^+$) by FACS from of whole splenic extracts from *wild type, relb*$^{-/-}$*, crel*$^{-/-}$*, and relb*$^{-/-}$*crel*$^{-/-}$ mice.

(b) Graphical representation of FACS plots is represented by scatter charts: *wild type* (●) *relb*$^{-/-}$(■) *crel*$^{-/-}$(▲) and *relb*$^{-/-}$*crel*$^{-/-}$(♦).

***In Vivo* analysis of double knockout mouse revels defective organization of the splenic marginal zone in mice lacking RelB and cRel.**

To support our findings from the previous section, defective B cell development in absence of RelB and cRel, we performed a histological analysis of the double knockout spleen first using HE stain. In wild type controls we can see strong separation of the white pulp (congregation site for lymphocytes) and red pulp (red blood cells). With the *relb*$^{-/-}$*crel*$^{-/-}$ spleen, the white pulp, while present, is not as dense, suggesting that lymphocyte (B cells) populations are reduced in this animal. These areas appear smaller than wild type controls (Figure 2.12). The reduction in white pulp could due to less B cells present in the spleen, as our FACS analysis would suggest. But it may also be a consequence of improper homing of the lymphocytes, and the cells could be dispersed throughout the spleen.

To address this possibility, we investigated the organization of B cells using immunofluorescence. Frozen sections of splenic tissue stained were anti-IgM and anti-IgD to identify the immature and mature B cell subsets respectively. MOMA-1 (which stains MZ metallophilic macrophages) was used to outline the follicular rim surrounding the B cell rich zone. In agreement with our FACS data, the presence of mature B cells was greatly diminished in the *relb*$^{-/-}$*crel*$^{-/-}$ spleen, as shown by the staining of anti-IgD (blue). We also note an increase in anti-IgM^{+} populations (green) in the mutant, however not seen in the proper formation within the follicles; but dispersed throughout the spleen

As we have shown, the *relb*$^{-/-}$*crel*$^{-/-}$ is devoid of MZ B cells (Figure 2.10), but a dearth of immature B cells, which makes us believe that the increased ant-IgM signal is due to the high immature B cells. Finally, MOMA-1 staining (red) revealed macrophage formation into the characteristic ring-like structure seen in the wild type controls, yet not apparent in the double knockout mouse. Our histology study informs us that the compound absence of RelB and cRel indeed lead to a loss of total B cells, as well as decreased population of mature B cells. Surprisingly, we also find that that splenic architecture of the *relb*$^{-/-}$*crel*$^{-/-}$ mouse is defective, which in turn may affect the homeostatic regulation of B cell development / survival in these mutant mice.

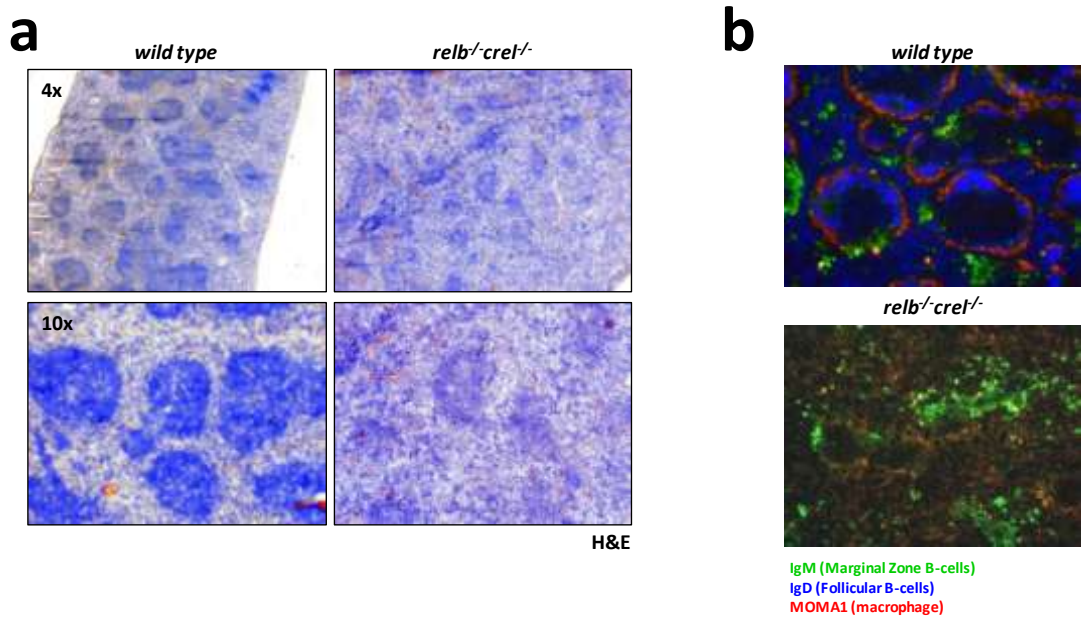

Figure 2.12. Defective organization of the splenic marginal zone in mice lacking RelB and cRel.
(a) Histological analysis of splenic sections taken from *wild type* and *relb*$^{-/-}$*crel*$^{-/-}$ mouse using hematoxylin and eosin stain (H&E).
(b) In vivo analysis of mature B cell development in double knockout mouse using immunofluorescence of frozen splenic sections stained with 1) anti-IgM FITC, 2) anti-IgD APC, and 3) anti-MOMA-1 Alexa Fluor® 568.
IF stains and photos performed in collaboration with D. Otero

B cell developmental defect displayed in *relb*$^{-/-}$*crel*$^{-/-}$ mouse is B cell intrinsic.

The histology result for the double knockout spleen raises an interesting contention; does the improper spleen architecture play a role on the defective B cell development? Within secondary lymphoid organs, such as the spleen, peripheral B cells become dependent on a diverse cytokine milieu, such as BAFF and APRIL, assisting in the proper B cell progression and maintenance (Mackay and Schneider, 2009)(Rickert et al., 2011). With the disruption of spleen infrastructure in the *relb*$^{-/-}$*crel*$^{-/-}$ mouse, this relationship may be ruined. We now ask if the developmental defect of B cells observed in double knockout mice is due to a cell intrinsic mechanism, or is the spleen environment incapable of sustaining a natural pool of recirculating B cells.

To do this we created mixed-bone marrow (BM) chimeras, where hematopoietic stem cells from the bone marrow of *wild type* mice are mixed with stem cells of mutant NFκB to a 50-50 ratio, then injected into a lethally irradiated wild type mouse. In order to follow the development of donor mice. To track donor hematopoietic cells following bone marrow transplantation, we utilized the CD45 congenic marker system. Donor mice carried either CD45.1 or CD45.2 marker, while contamination of recipient mice following transplantation could be determined with the CD45.1.2 marker (Figure 2.13).

With this system in place, we can determine whether the *relb*$^{-/-}$*crel*$^{-/-}$ (CD45.2) hematopoietic stem cells can develop properly when given the

advantage of a normal splenic structure, along with growth factors / cytokines provided by the *wild type* (CD45.1) donors. Our examination of cRel and RelB deficient controls show a similar pattern observed from the normal FACS experiments (Figure 2.9), where the mature B cell development is stunted in RelB and cRel deficient donors, resulting in a reduction of follicular and marginal zone B cells. Again, the immature B cell populations are higher than the *wild type* controls. We can also see that the T1 immature B cells have nearly 3x fold larger proportion in *relb*$^{-/-}$*crel*$^{-/-}$ chimeras (Figure 2.14). Unlike the *relb*$^{-/-}$*crel*$^{-/-}$ donors, the *wild type* controls all develop normally in the recipient mouse. As a reference, we also created chimeras from RelB and cRel single knockout mice, with no observable phenotype (Figure 2.14). Our conclusion from this chimera study is the B cell developmental phenotype seen in RelB cRel double knockout mouse is due to a B cell intrinsic mechanism, and not caused by the improper spleen architecture.

To further demonstrate that the defective spleen organization is capable of sustaining normal B cell maintenance, we produced a second mixed mouse chimera in which *wild type* and *relb*$^{-/-}$*crel*$^{-/-}$ donor bone marrow stem cells were injected into lethally irradiates *relb*$^{-/-}$*crel*$^{-/-}$ recipient mice. The outcome of this experiment was that *wild type* donor stem cells were able to mature routinely from T1 to T2, FO, and MZ B cells (Figure 2.15), further substantiating our conclusions that the spleens of these *relb*$^{-/-}$*crel*$^{-/-}$ mice are qualified of

sustaining proper B cell developmental, and the B cell defect of the animals is cell intrinsic.

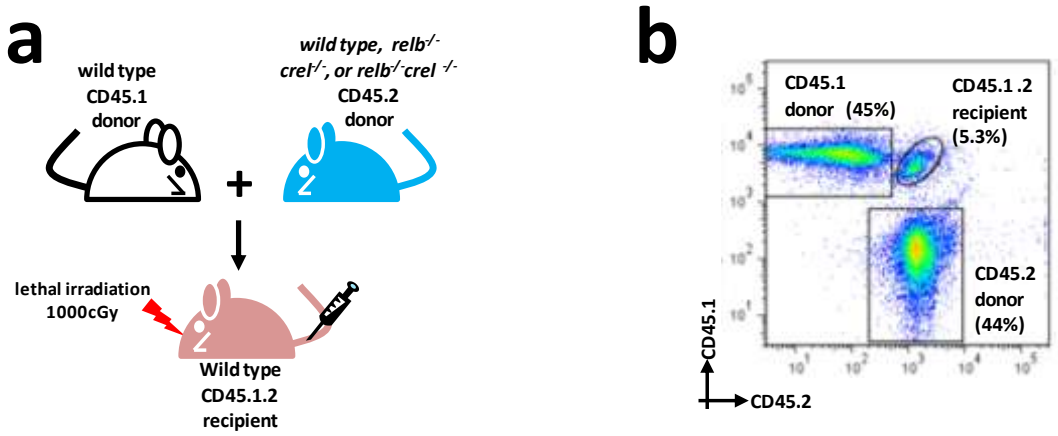

Figure 2.13. Experimental design of mixed mouse chimeras.
(a) Bone marrow stem cells from CD45.1 *wild type* and CD45.2 wild type, *relb*$^{-/-}$, *crel*$^{-/-}$, or *relb*$^{-/-}$*crel*$^{-/-}$ are mixed in a 50 / 50 ratio, and then injected into a lethally irradiated CD45.1.2 *wild type* mouse.
(b) Representative FACS analysis of the mixed mouse chimera 8 weeks following the stem cell transplant. Using CD45 as a marker, identification of both donors and recipient can be obtained.
Tail vein injections performed by E. Yang.

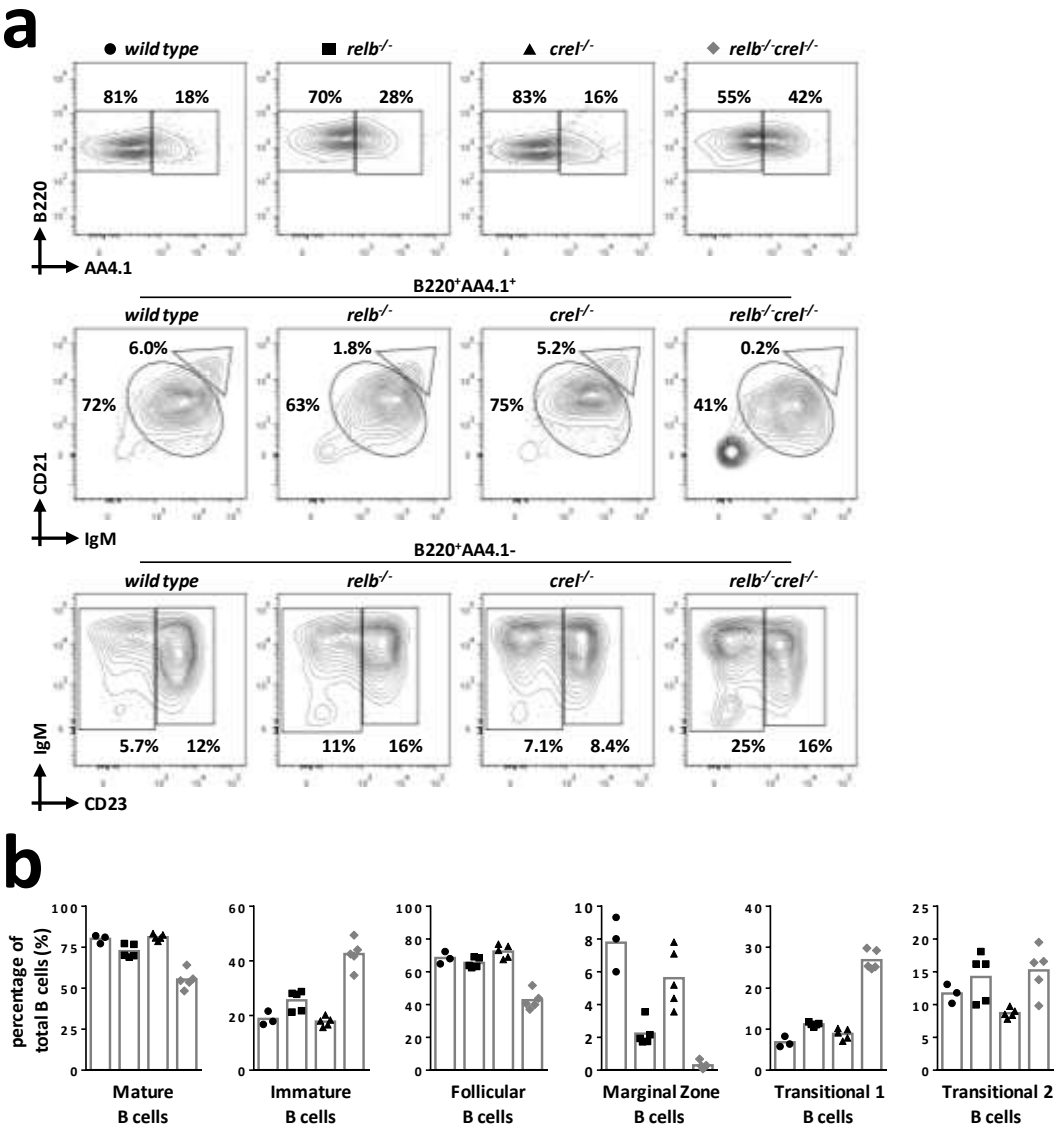

Figure 2.14. B cell developmental defect displayed in *relb*$^{-/-}$*crel*$^{-/-}$ mouse is B cell intrinsic.

(a) Representative FACS plots of mixed-chimeras outlined in figure 3.10. Top panel: Identification of mature (B220^{+}AA4.1^{-}) and immature B cells (B220^{+}AA4.1^{+}). Middle panel: mature follicular B cells (CD21^{+}IgM^{+}) and mature marginal zone B cells (CD21^{high}IgM^{+}) Bottom panel: transitional one (T1) B cells (CD23^{-}IgM^{+}) and transitional two (T2) B cells (CD23^{+}IgM^{+}).

(b) Graphical representation of FACS plots is represented by scatter charts: *wild type* (●) *relb*$^{-/-}$(■) *crel*$^{-/-}$(▲) and *relb*$^{-/-}$*crel*$^{-/-}$(♦).

Tail vein injections performed by E. Yang.

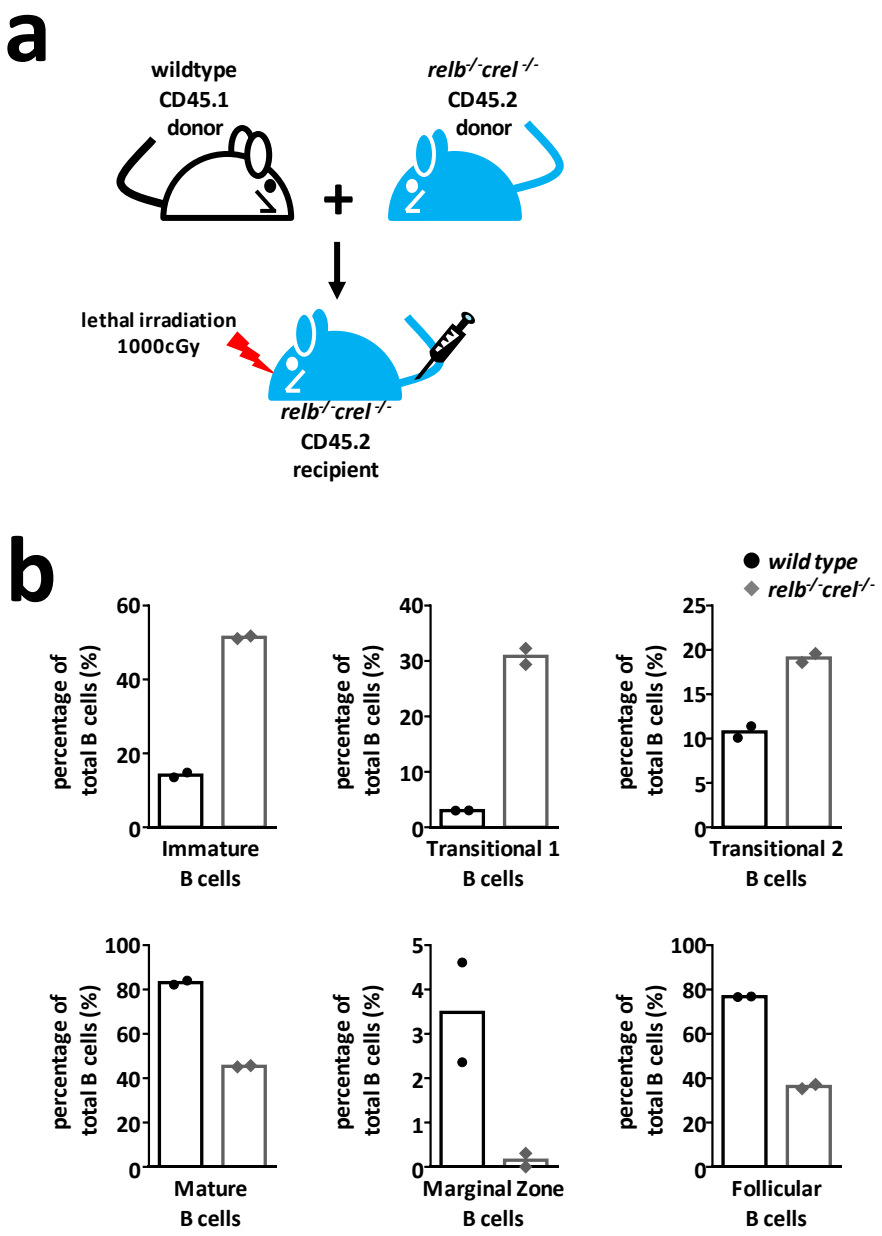

Figure 2.15. Splenic environment of relb-/-crel-/-mouse is capable of supporting normal B cell development.

(a) Bone marrow stem cells from CD45.1 *wild type* and CD45.2 *relb*$^{-/-}$*crel*$^{-/-}$ are mixed in a 50 / 50 ratio, then injected into a lethally irradiated CD45.2 *relb*$^{-/-}$*crel*$^{-/-}$ mice.

(b) Graphical representation of FACS plots is represented by scatter charts: *wild type* (•) and *relb*$^{-/-}$*crel*$^{-/-}$ (♦).

Tail vein injections performed by E. Yang.

BAFF promotes B cell metabolic fitness in early gene profile and cell cycle progression at later time points.

Our biochemical analysis shows that BAFF stimulation will activate RelB and cRel NFκB family members. Deletion of both RelB and cRel results in a B cell developmental defect at the T1 transitional stage, which mimics a similar phenotype observed in BAFF deficient mice. NFκB is key transcription factor implicated in expression of proliferative and survival programs (Hayden and Ghosh, 2008)(Gerondakis and Siebenlist, 2010). We have shown that expression of pro-survival genes bcl-xl and A1 are dependent on cRel (Figure 2.7). We now would like to identify the gene programs responsible for BAFF mediated development and maintenance. Previous reports on BAFF induced genes used microarray technologies (Patke et al., 2006), but for this study we will employ RNA-sequencing (RNA-seq) which allows quantitative measurement of genome-wide expression levels of genes and their transcripts (Nagalakshmi et al., 2008).

Wild type B cells were stimulated with BAFF for two days in culture, and RNA was isolated at eight and thirty hours. Eight hours being near the peak of cRel activity, and 30 hours was selected to correspond with the activation of RelB in the absence of cRel. We aligned our reads were aligned to the mouse mm10 genome and RefSeq genes (PMID 12045153, PMID 12466850) with Tophat. With the unstimulated time point as our reference, we then identified

genes that were increased or decreased by two fold in at least one of the two time points to generate the represented heatmap (Figure 2.16).

From this study, we have found 499 genes with 2x fold less expression, or "repressed" genes in either time point, with the majority of the repressed genes occurring at the early time point with 457 genes. We find that at with persistent stimulation of BAFF has less repression (289).

Next we turn to gene profiles induced following BAFF stimulation; where we see 254 genes induced following eight hours of stimulation, and 347 genes are turned on with persistent BAFF signaling. Overall, 471 genes are induced 2x fold through the entire time course. Examination of induced gene groups terms and their associated biological processes was achieved using the online gene ontology tool, GORilla (Eden et al., 2009)(Eden et al., 2007). Previous studies implicated BAFF activation in gene transcription that direct these cells toward production of proteins required for glycolytic metabolism and cell cycle progression (Patke et al., 2006).Our analysis also shows the increase of genes involved in metabolic processes following the initial stimulation (Figure 2.17a.) however with constant BAFF signaling (30 hours) we see a shift in the programs toward cell cycle (Figure 2.17b). The implication of these results would suggest that a primary role of BAFF is to prepare B cells for a state of immediate responsiveness to antigenic stimulation (Patke et al., 2006).

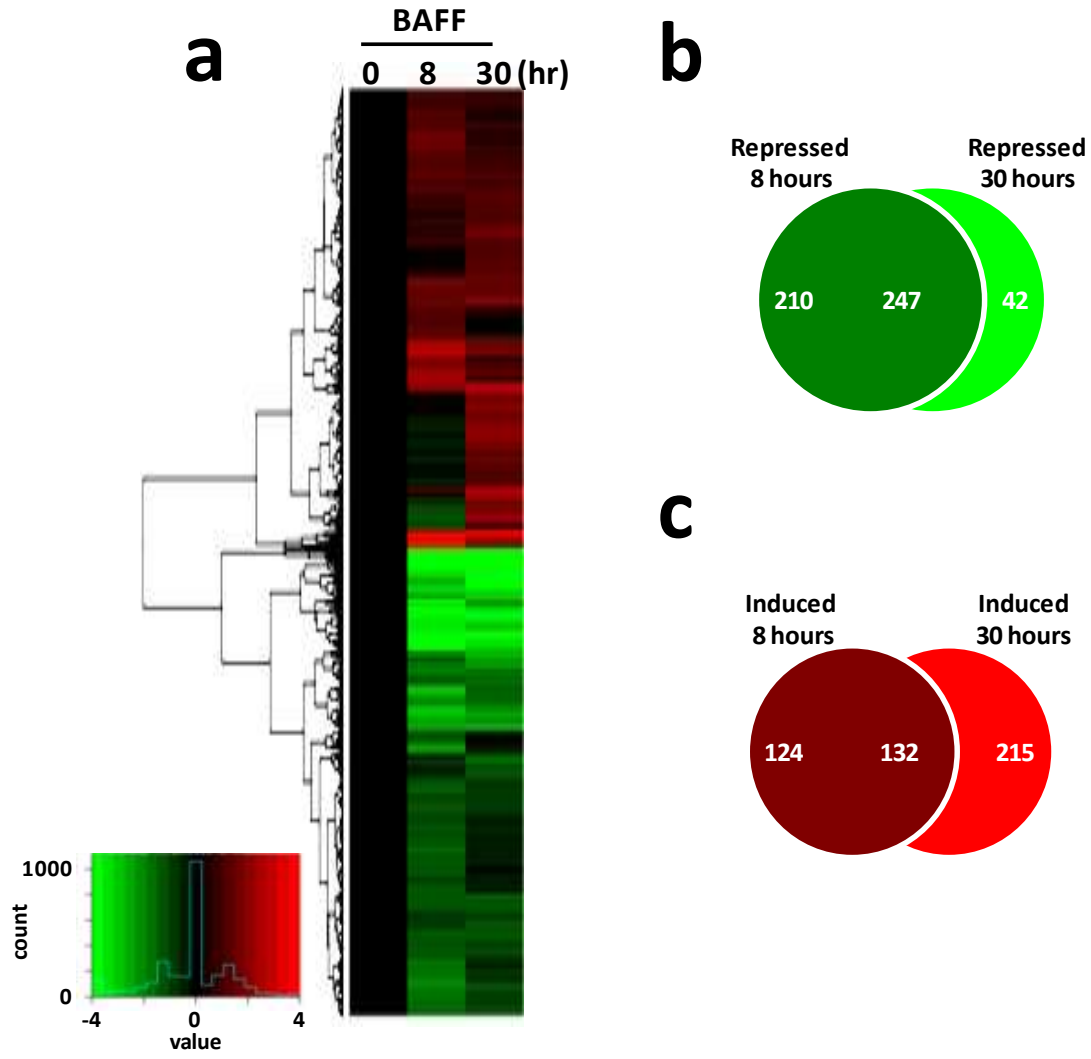

Figure 2.16. Gene expression profiles of BAFF stimulated *wild type* B cells.
(a) RNAseq analysis from *wild type* B cells stimulated with 50ng/ml BAFF at the indicated time points. Heatmap representing the expression pattern of genes containing a 2-fold increase or decrease in gene induction over the unstimulated state (0hr).
(b) Vin diagram of genes which show a 2x fold decrease in expression following BAFF stimulation.
(c) Vin diagram of genes which show a 2x fold increase in expression following BAFF stimulation at the indicated time points.
RNAseq analysis performed in collaboration with H. Birnbaum and J. Davis-Turak.

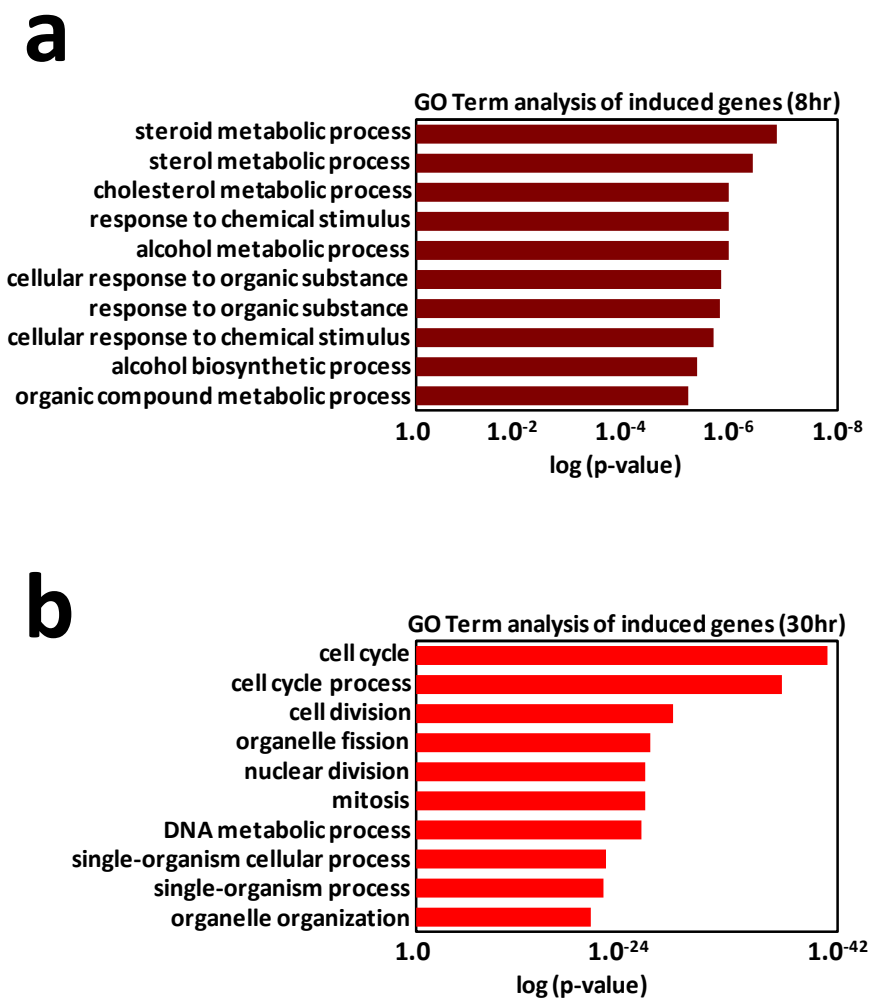

Figure 2.17. BAFF promotes B cell metabolic fitness in early gene profiles and cell cycle progression at later time points.
(a) Go term analysis of all 256 genes induced greater than 2x fold (log2) following eight hours of BAFF stimulation.
(b) Go term analysis of all 347 genes induced greater than 2x fold (log2) following thirty hours of BAFF stimulation.

Future directions: What are the of RelB and cRel dependent genes in BAFF mediated survival / development?

The role of non-canonical NFκB signaling in BAFF activation has been explored in numerous reports(Claudio et al., 2002)(Fu et al., 2006)(Kayagaki et al., 2002), and it's association in BAFF survival has been recognized in this chapter (Figure 2.6), yet identification of the RelB-controlled gene products responsible for BAFF function has not been elucidated. BAFF activation leads to transcription of pro-survival genes like activate transcription of B cell CLL/lymphoma 2 (Bcl-2) and/or BCL2-like 1 (Bcl-XL) (Claudio et al., 2002)(Craxton et al., 2005). However, we have shown that RelB is not required in the expression of Bcl-XL, while cRel does (Figure 2.7). Our survival assay has shown that canonical mediator cRel and non-canonical RelB are involved in BAFF *in vitro* survival, along with the biochemical data displaying activation or RelB and cRel following BAFF stimulation. Current literature has pointed to a few key genes that are induced by BAFF, yet there still remains a lack of information regarding which genes are controlled by RelB, and even cRel.

In the immediate future, we have proposed to identify the genes that cRel and RelB responsible for mediating BAFF functions in B cell survival and development. For this we shall perform chromatin immunoprecipitation sequencing using anti-RelB and anti-cRel antibodies on B cell stimulated with BAFF for eight and thirty hours. From this approach we hope to classify the

gene programs governed by NFκB into three categories: 1) RelB-dependent, 2) cRel-dependent, and 3) shared genes (Figure 2.18).

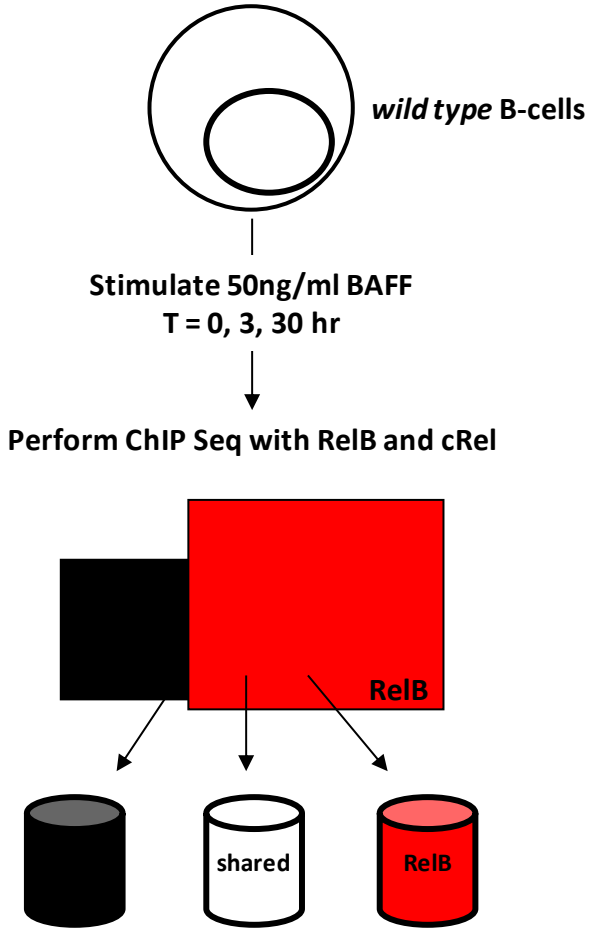

Figure 2.18. Identification of RelB- and cRel-dependent genes in BAFF signaling.
Schematic of planned experiment to pinpoint the genes which are controlled by RelB and cRel during BAFF stimulation.

Discussion

For over a decade, numerous reports have focused on characterization of non-canonical NFκB roles in the BAFF signaling pathway, yet few have come to recognize the importance of canonical NFκB family members in controlling BAFF functions (Hatada et al., 2003)(Sasaki et al., 2006)(Enzler et al., 2006). In this study we find that cRel and RelA, NFκB family members typically characterized as canonical NFκB effectors, to be activated following BAFF stimulation. Further, we see that the kinetics of such activation result in a rapid spike in RelA-containing species soon followed by a more persistent cRel activity. This profile is consistent with routine NEMO-IKK1/IKK2 canonical signaling, yet we observed little to no NEMO activity via *in vitro* kinase assay. In addition, we found BAFF has no effect on IκBα degradation, suggesting that the mechanism of BAFF mediated RelA and cRel release may be divergent from other B cell stimuli like anti-IgM or LPS.

Nevertheless, cRel activity does play a role in BAFF functions, as cRel deficient B cells response to BAFF survival signals *in vitro* weakly compared to *wild type* controls. As expected, *relb*$^{-/-}$ B cells quickly died in culture despite the presence of BAFF. However, compound deletion of both RelB and cRel resulted in an even more dramatic survival defect than the single knockouts alone. This defect is in part due to loss of cRel dependent expression of pro-survival genes A1 and bcl-XL, yet the RelB-dependent genes are yet to be discovered.

BAFF is critical for the development and maintenance of peripheral B cells. Given the biochemical and in vitro results, we looked at the effect of removing RelB and cRel in B cell development. We find that double deletion of RelB and cRel result in a blockage in splenic B cell development at the transitional 1 to 2 stage. These mutant mice possess an two-fold increase in the percentage of immature B cells, and almost four-fold enhancement in the population of T1 cells. This observation at T1 to T2 stage mimics the phenotype of BAFF knockout mice; however the defect is not as severe, possibly due to compensation of RelA, or some other NFκB-independent mechanism. As a result of this early stage deficiency, we find that the mature B cells *relb*$^{-/-}$*crel*$^{-/-}$ mice are diminished, with no detectable marginal zone B cells and a marked decrease in follicular B cell numbers. This developmental defect was also shown to be B cell intrinsic, as *relb*$^{-/-}$*crel*$^{-/-}$ bone marrow stem cells failed to develop normally in mixed-bone chimeras. To further support this observation, we showed that wild type hematopoietic stem cells are capable of proper B cells development in lethally irradiated *relb*$^{-/-}$*crel*$^{-/-}$ mice, despite an improper splenic architecture present in the mutant animals. In sum, we have illustrate the importance of RelB and cRel effectors in BAFF functions.

BAFF ligand is capable of binding three TNF receptors; 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)(Mackay and Schneider, 2009) . With these three possible binding receptors, dissection of the BAFF signaling pathways becomes difficult. Luckily, BCMA expression is not found on the typical peripheral B cells subsets (Stadanlick and Cancro, 2008), leaving only TACI and BAFF-R as the two mediators of BAFF activation. TACI however can activate canonical NFκB (Mackay and Schneider, 2008), thus we must appreciate it's contributions to our analysis. With that said, TACI-deficient B cells are capable of degrading Iκbα in response to BAFF similarly as do wild type control B cells (Shinners et al., 2007) , therefore BAFF-R signaling alone can activate classical NFκB.

This same report also explored a similar topic of classical NFκB in BAFF-R signaling. Shinners et al. implicated BAFF-R signaling via Bruton's Tyrosine Kinase (btk), a critical protein normally associated with downstream BCR effects. Similar to our results, they show BAFF stimulation leads to activation of RelB and cRel containing dimers by EMSA. And both family member s activities are repressed in the absence of btk. They showed that deletion of BTK would lead to less survival via BAFF, however they did not discern if this was dependent on less cRel activity, while our data clearly shows diminished BAFF survival *in vitro* in *crel*$^{-/-}$B cells.

In contrast to the mechanism proposed by Shinners et al, we did not see activation of canonical NFκB in a NEMO-dependent manner (Figure5a). Nor did we see any IκBα degradation following stimulation of wild type B cells,

which would be consistent with the release of RelA typical of canonical NFκB kinetics (Figure 2.5b). We examined the degradation at 1hr, which would coincide with the peak of RelA activity observed at 1hr in our EMSA (Figure 2.1). IκBα degradation is a method which was believed to be a BAFF can activate cRel containing dimers (Shinners et al., 2007).

This discrepancy may be a result of different BAFF sources; their BAFF source is recombinant human BAFF purified from Chinese hamster ovary cells (Thompson et al., 2001), our BAFF is a recombinant mouse variety commercially available from R&D systems (see materials and methods).Soluble BAFF can exist as a homotrimers or as a capsid-like assembly of 20 trimers (60-mer) (Mackay and Schneider, 2009). Indeed, activation of canonical NFκB via TACI oligomerization is dependent on the type of BAFF available (Bossen et al., 2008; Chang et al., 2006). Perhaps the same can be said of BAFF-R, as the BAFF ligand's species origin and composition may affect the kinetics and even still the mechanism by which NFκB is turned on.

Shinners et al. clearly showed that cRel is activated by BAFF stimulation. However, unlike our study, they did not examine the role of RelA in the same capacity. Activation of RelA, as in the case of LPS or TNF stimulation, occurs in a NEMO-dependent manner resulting in IκBα degradation and release of functional RelA dimers. Activity is typically rapid and transient, which is what we observed with BAFF (Figure 2.1). In line

with our observations of little to no NEMO activity and stable IκBα levels following BAFF introduction, we consider an alternative mechanism of RelA and cRel activation. Our lab previously reported stimulation of lymphotoxin-β, like BAFF-R a member of the TNF superfamliy, can prompt IKK1-dependent inactivation of a fourth IkB, termed Iκbδ, allowing for activation of the RelA:p50 dimer (Basak et al., 2007).

Outlined in Figure 2.19b, we propose BAFF stimulation results in activation of non-canonical NFκB in a NIK-IKK1 dependent manner, resulting in the release of effectors RelB-, RelA-, and cRel-containing dimers. Our model includes the established understanding by which RelB:p52 activity occurs; a result of p100 processing to p52 leading to the expression of gene programs, including survival (Claudio et al., 2002). Our model offers an alternative manner in which RelA / cRel becomes activated. Latent RelA and cRel containing- dimers are trapped by Iκbδ in naive, resting B cells. NIK-IKK1 complex becomes activated with the addition of BAFF, leading to the degradation of IκBδ and release of RelA. Active RelA dimers translocate to the nucleus to switch on the expression of cRel. The intensity of BAFF-induced cRel activity is highest ~3 hours, which follows the peak of RelA activity (Figure 2.2), suggesting that the dynamics of pre-existing cRel dimers is slower than RelA or more likely, the primary source of observed cRel activity is due to newly synthesized cRel induced by RelA.

As of this moment, further biochemical analysis of BAFF mediated NFκB activation is required to validate our model. It would be remiss to say that a NEMO-dependent mechanism does not exist. In fact, both methods may be non-exclusive. What our study does demonstrate is survival and development by BAFF requires multiple NFκB family members, namely RelB and cRel. Deletion of both these family members results in defects *in vitro* and *in vivo.* The current responsibilities of RelB and cRel in BAFF biology are still unclear. Currently we are examining these functions using genome-wide bioinformatic techniques with the hopes of delineating the distinct and overlapping roles of RelB, cRel and even RelA.

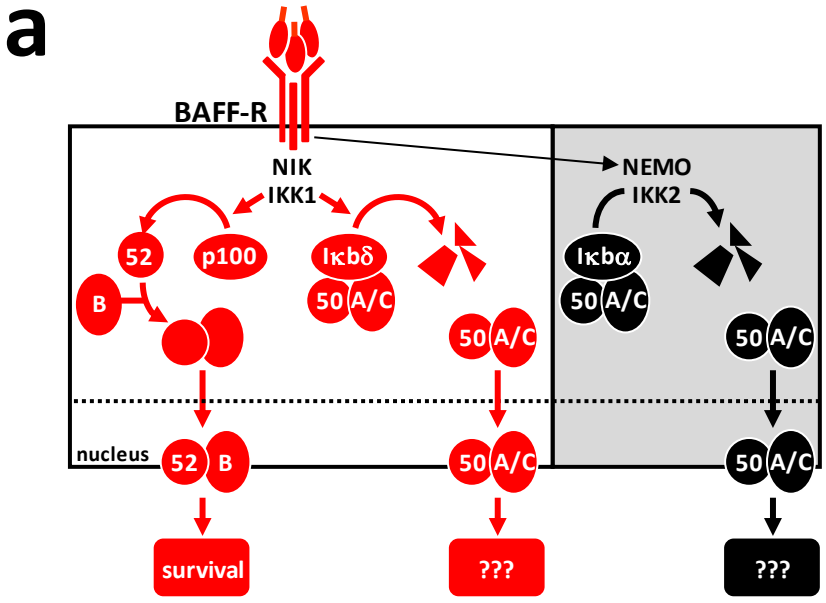

Figure 2.19. Mechanisms for BAFF activation of NFκB.
(a) BAFF can activate both canonical and non-canonical NFκB pathways; a NEMO-dependent activation resulting in degradation of classical IκBα leading to the release of RelA and cRel effectors (shown in black, right shaded box). In addition, it triggers NIK dependent non-canonical NFκB pathways leading to p100 processing into p52 ultimately resulting in RelB:p52 nuclear localization. However, NIK activity may also be responsible for the activation of RelA and cRel containing dimers through targeted degradation of Iκbδ, analogous to IκBα, releasing latent RelA:p50 and cRel:50 dimers.

Materials and Methods

Cell isolation and culture. Spleens were harvested from C57Bl6 mice *wild type* (Jackson Labs, Bar Harbor, MN). The collected spleens were homogenized using frosted glass slide grinding. For B cell isolation, homogenized splenocytes were incubated with anti-CD43 (Ly-48) microbeads for 15 minutes at room temperature. Following this incubation, the cells were washed with Hanks Buffered salt solution (HBSS) (Gibco 14170) containing 1% FCS, 10mM HEPES (Gibco 15630) and 1% FCS (Sigma F2442) and separated over a magnetic column (LS column MiltenyiBiotec 130-042-401). For B cells, purity was determined by flow cytometry using PE anti-B220 (eBioscience 12-0452-83), FITC anti-CD3 (eBioscience 11-0031-82), APC anti-CD4 (eBioscience 17-0041-83) and PerCP anti-CD8 (ebioscience 46-0081-82). Purity was consistently found to be between 92% and 95% (data not shown). Complete media consisting of RPMI-1640 (Gibco 11875), 10mM HEPES (Gibco 15630), 1 mM Sodium Pyruvate (Gibco 11360), 1 mM non-essential amino acids (Gibco 11140), 0.055 mM β-mercaptoethanol (Gibco 21985), 100 units Penicillin/Streptomycin (Gibco 10378016) and .3 mg/ml glutamine was used to culture either B cells. B cells were stimulated with 50ng/ml Recombinant Mouse BAFF / BLyS / TNFSF13B (R&D systems 2106BF).

Electrophoretic Mobility Shift Assay. Nuclear extracts were generated from B cells using high salt extraction. In brief, purified B cells were

incubated with a low salt buffer (10mM HEPEs pH 7.9 (Gibco), 10mM KCl (Thermo Fisher Scientific P217), 0.1mM EGTA (Sigma E-4378), 0.1mM EDTA (Thermo Fisher Scientific S312), 1mM DTT (Thermo Fisher Scientific BP172-5), 1mM PMSF (Sigma P7626), 5μg/ ml apoprotein (Sigma A1153), 5μg/ml leupeptin (Sigma L2884), 1μM pepstatin A (Sigma P5318) for 10 minutes on ice. Following this incubation, the cells were disrupted through the addition of NP-40 (US Biological N3500) to a final concentration of .5% and vortexing for 15 seconds. Nuclei were pelleted away from the cytoplasmic fraction by centrifugation at 15,000 rpm for 1 minute and the cytoplasmic fraction was pipetted into a separate tube. The remaining nuclei were disrupted by a 20 minute incubation at 4oC in a high salt buffer (20mM HEPES pH 7.9 (Gibco), 400mM NaCl (Thermo Fisher Scientific S671), 1mM EGTA (Thermo Fisher Scientific), 1mM EDTA (Thermo Fisher Scientific), 20% Glycerol (Thermo Fisher Scientific), 1mM DTT (Thermo Fisher Scientific), 1mM PMSF(Sigma)). The nuclear fraction was collected following centrifugation at 15,000 rpm for 5 minutes. Equal amounts of nuclear extracts (1ug) were pre-incubated for 20 minutes on ice in the presence or absence of antibodies specific for RelA (Santa Cruz Biotechnology sc-372), RelB (Santa Cruz Biotechnology sc-226), and cRel (Santa Cruz Biotechnologies sc-71) or in combination. Following the pre-incubation with antibodies, [γ–32P]ATP (GE health) radio-labeled probe derived from HIV-κB sequence:

5’-GCTACAAGGGACTTTCCGCTGGGGACTTTCCAGGGAGG-3’ was added and incubated at room temperature for an additional 15 minutes. The resulting DNA/protein/antibody complexes were resolved by electrophoresis on a 5% non-denaturing polyacrylamide gel and exposed to storage phosphor screen (GE healthcare) overnight before image development on at Typhoon 9200 Variable Mode Imager (GE healthcare). Images were analyzed and quantitated using ImageQuant™ (GE Health).

FACS antibodies. 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), AA4.1,and IgM (115-096-020; Jackson Immunoresearch).

Flow cytometry analysis of survival and B cell development. Purified B cells were stained with cultured in complete media with or without BAFF, at various time points B cells were collected and stained with 7AAD (Invitrogen A1310). The B cells were analyzed for survival using a C6 Accuri flow cytometer (BD Biosciences). Differences in cell viability was measured using FlowJo (Tree star inc.).

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) and analyzed using FlowJo software (Tree Star, Inc.).

Bone marrow chimeras. Donor bone marrow cells were isolated from femora of *wild type, crel*$^{-/-}$*, relb*$^{-/-}$ *or relb*$^{-/-}$*crel*$^{-/-}$ mice and injected (~5x10^6 cells intravenously per mouse) into 2- to 3-month-old C57BL/6 recipients. Before injection, recipient mice were lethally irradiated recipient mice (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.

RNAseq. Total RNA was isolated from 50ng /ml BAFF stimulated wild type B cells isolated following 8 and 30 hours of culture. mRNA was extracted from 2 µg total RNA using oligo(dT) magnetic beads and fragmented at high temperature using divalent cations. cDNA libraries were generated using the Illumina TruSeq 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 by the BIOGEM core facility located at 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 CummRbund (31) was used to ascertain differential expression of genes. Gene differential FPKMs were obtained from the cuffdiff program in the Tuxedo RNA-seq analysis suite.

Acknowledgements

RNAseq alignment was done by Harry Birnbaum and Jeremy Davis-Turak. RNAseq analysis was shared collaborative effort with the thesis author and Harry Birnbaum and Jeremy Davis-Turak. Bone marrow chimeras are a collaborative effort with Edward Yang, Masataka Asagiri, and Ananda Goldrath.CD45.1CD45.2 donor mice were provided by the Goldrath lab. Louise Delacruz irradiated the donor mice. . Masataka assisted in the bone marrow extraction. Edward performed all tail vein injections. Spleen removal, cell isolation, FACS staining, and analysis was performed by myself. Frozen spleens for immunofluorescence (IF) was done by the UCSD histology core at Moores Cancer Center. Dennis Otero and I collaborated for IF of frozen spleen sections; including staining, taking pictures, and analysis. This work is currently unpublished. I am the primary author. Jeremy Davis-Turak, Harry Birnbaum ,Masataka Asagiri, Edward Yang, Dennis Otero and Ananda W. Goldrath are co-authors. Alexander Hoffmann is the corresponding author.

CHAPTER 3:

BAFF superactivates cRel-driven B cell expansion by neutralizing the IκBsome.

Abstract

BAFF-R has critical roles in providing survival signals during B cell maturation and in contributing to B cell proliferation. Here we show BAFF-R survival functions in resting B cells is in large part dependent on the NFκB transcription factor RelB, a well-known effector of the non-canonical pathway. In contrast, BAFF-R's contribution to antigen-responsive B cell expansion is not mediated by RelB or enhanced survival, but by cRel determining the fraction of cells committing to the cell division program. However, BAFF-R controls these distinct transcriptional effectors via the same so called "non-canonical" NFκB pathway: in maturing B cells BAFF-R enhances p100 processing to p52 to generate the RelB:p52 dimer, but during BCR-responsive proliferation, inactivation of the p100-containing multimeric IκBδ activity, which can inhibit cRel, is the functionally relevant molecular mechanism. Analysis of BCR and BAFF-R co-stimulated B cell transcriptome detected a cRel-signature. These experimental observations were recapitulated by a mathematical model of NFκB dimer generation and IκB-mediated control of their activity. Using nfkb2$^{+/-}$ B cells, we were able to validate a critical model-derived hypothesis that a reduction in p100 expression may phenocopy BAFF co-stimulation to achieve full B cell expansion. In sum, our studies revealed that BAFF-R achieves its physiological functions through two distinct transcriptional effectors RelB and cRel that are downstream of the NFκB non-canonical pathway.

Introduction

B cell expansion is achieved through the regulation of cell division and survival. Mature follicular B cells are largely responsible for thymus (T)-dependent antigenic responses. Two receptors critical in the process of maintenance and activation of the follicular B cell pool are the B cell antigen receptor (BCR) and the B cell activating factor (BAFF) receptor (Cancro, 2009; Khan, 2009). NFκB signaling through these receptors is known to be mediated by two distinct pathways: the NEMO-dependent “canonical” pathway, and the NEMO-independent “non-canonical” pathway. Activated BCR recruits the CBM complex to the membrane, as well as TRAF2 and TRAF6, causing NEMO ubiquitination and activation of the NEMO-containing IKK complex. This leads to nuclear translocation of pre-existing or latent RelA- and cRel-containing NFκB dimers (Hayden and Ghosh, 2008). BAFF-R stimulation triggers sequestration of via TRAF3, resulting in the stabilization of NIK and activation of a NEMO-independent IKK1 kinase complex. This enhances p100 processing to p52, and nuclear accumulation of RelB:p52 dimers (Claudio et al.).

Inducible ablation of the BCR leads to a rapid loss in mature B cell totals, despite the presence of functional BAFF-R (Lam et al.). In turn, BAFF 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(Harless et al., 2001;

Schiemann et al., 2001). This would suggest that B cell development and maturation are reliant on signals from both the BAFF-R and BCR. Genetic ablation of NFκB family members result in partial of complete loss of peripheral B cell subsets indicating the critical role of NFκB in B cell development and maintenance (Claudio et al., 2002; Grossmann et al., 2000).

The interdependence or integration of BAFF-R and BCR signaling has been the focus of several recent studies. One underlying mechanism suggests that the two receptors signaling pathways share key signaling components. Syk tyrosine kinase, a known transducer of BCR activity was shown to activate key BAFF-R survival signals (Schweighoffer et al., 2013). Deletion of Btk, another crucial BCR signal transducer, diminishes BAFF-mediated survival *in vitro*(Shinners et al., 2007). A second model posits that for B cell maintenance, tonic BCR signals are a pre-requisite for the BAFF-R to transmit survival signals; specifically, tonic BCR-dependent canonical NFκB signaling raises constitutive RelA- and cRel-containing dimer activities, which induce expression of the nfkb2 gene to provide a pool of p100 substrate for sustained IKK1-dependent processing to p52 and production of the known non-canonical effector RelB:p52(Cancro, 2009; Stadanlick et al., 2008). A similar dependency between canonical and non-canonical signaling was found in the response to lymphotoxin beta (Basak 2008). RelB is thus the presumed mediator of BAFF's survival functions and BAFF-induced anti-apoptotic gene expression, though its genetic requirement remains to be established.

In addition to BAFF's essential role in the maturation and survival of juvenile peripheral B cells, during antigen-stimulation of mature follicular B cells co-stimulation of BAFF-R results in enhanced B cell expansion *in vitro*(Huang et al., 2004; Rickert et al., 2011; Schweighoffer et al., 2013). Indeed, BAFF has been implicated in plasma cell survival (O'Connor et al., 2004), and it enhances both T cell-dependent and independent humoral immune responses (Do et al., 2000; Litinskiy et al., 2002). While formation of germinal centers (GC) was found to be independent of BAFF, the B cell responsiveness to antigens is impaired in *BAFF*$^{-/-}$ mice (Rahman et al., 2003; Vora et al., 2003). Extending the aforementioned model to BAFF's function as a co-stimulus in antigen-triggered B cell expansion, predicts that in these conditions BAFF produces increased amounts of RelB activity that enhances cell survival to augment B cell population dynamics. However, there are indications that BAFF may in fact contribute to cell cycle entry of mature follicular B cells, the primary responders to T cell dependent activation, following antigenic stimulation(Allman et al., 2001; Do et al., 2000; Huang et al., 2004; Patke et al., 2006). It is unknown whether this function may also involve NFκB signaling or be entirely mediated by other signaling axes known to be activated by BAFF, such as PI3kinase(Mackay and Schneider, 2009; Mackay et al., 2007;Rickert et al., 2011).

Here, we addressed the role of the NFκB signaling system in mediating BAFF's functions in B cells using reverse genetics, quantitative cell biology

and biochemistry, and mathematical modeling. In particular, we report that RelB is indeed critical for BAFF-induced survival of maturing B lymphocytes, but that BAFF's enhancement of the BCR-triggered population expansion is not based on enhanced survival or RelB. Instead, BAFF co-stimulation enhances BCR-triggered cRel activation and the fraction of B cells entering the proliferative program. Quantitative analysis of the signaling network reveals that cRel hyperactivation is mediated by the non-canonical pathway, which neutralizes the inhibitory effects of p100 overexpression that leads to the recently described multimeric IκBsome(Savinova et al., 2009)and its associated IκBδ activity (Basak et al., 2007; Shih et al., 2009). Encoding these signaling processes in a new mathematical modeling framework, we show that they are sufficient to account for the observations, and develop several new predictions that are then tested and confirmed experimentally.

Results

BAFF-R enhances BCR-triggered B cell expansion

Prior work has established that BCR and BAFF-R activate distinct NFκB signaling pathways, the so-called canonical, NEMO-dependent pathway, and the so-called non-canonical, NEMO-independent pathway, respectively (Figure 3.1a). Whereas the primary transcriptional effector of the former are cRel-containing NFκB dimers which may initiate the B cell proliferative program and enhance survival, the latter is known to activate RelB as well as other signaling pathways (e.g. PI3K) to enhance survival (Mackay et al., 2007; Ramakrishnan et al., 2004; Zarnegar et al., 2004).

We asked whether BAFF co-stimulation would enhance BCR-triggered B cell expansion via enhanced cell survival, or whether other signaling crosstalk mechanisms might be involved. Using the dye dilution with Carboxyfluorescein succinimidyl ester (CFSE), we monitored splenic B cell expansion following BCR cross-linking with anti-IgM [5-10 μg/ml, or molar equivalents of the F(ab′)2 fragment]. Co-stimulation with BAFF-R increased this B cell expansion, as first observed at 44 hrs and confirming previous reports (Craxton et al., 2007; Huang et al., 2004; Schweighoffer et al., 2013; Stadanlick et al., 2008)(Figure 3.1b).To determine how BAFF affected B cell population dynamics, we employed the software tool FlowMax (Shokhirev and Hoffmann, 2013), which deconvolutes dye dilution data to determine the fraction of cells entering the proliferative program (pF) and the associated cell

division times (Tdiv), and cell survival times (Tdie) of cells not (yet) proliferating. This automated interpretation of CFSE data, showed that BAFF not only increased the life time of B cells (from 40 to 61 hrs), but substantially increased the fraction of responding cells entering the proliferative program from 5 to 15% (Figure 3.1c). However, the time required till the first cell division remained constant at 43-48hrs. We confirmed this interpretation using 7AAD staining which revealed a greater percentage of live cells and far fewer that are 7AAD positive at 24hrs (Figure 3.2d), but in a longer timecourse the number live cells was dramatically enhanced with BAFF(Figure 3.2e) consistent with previous reports that BAFF is able to contribute to cell cycle entry as well (Allman et al., 2001; Do et al., 2000; Huang et al., 2004; Patke et al., 2006).

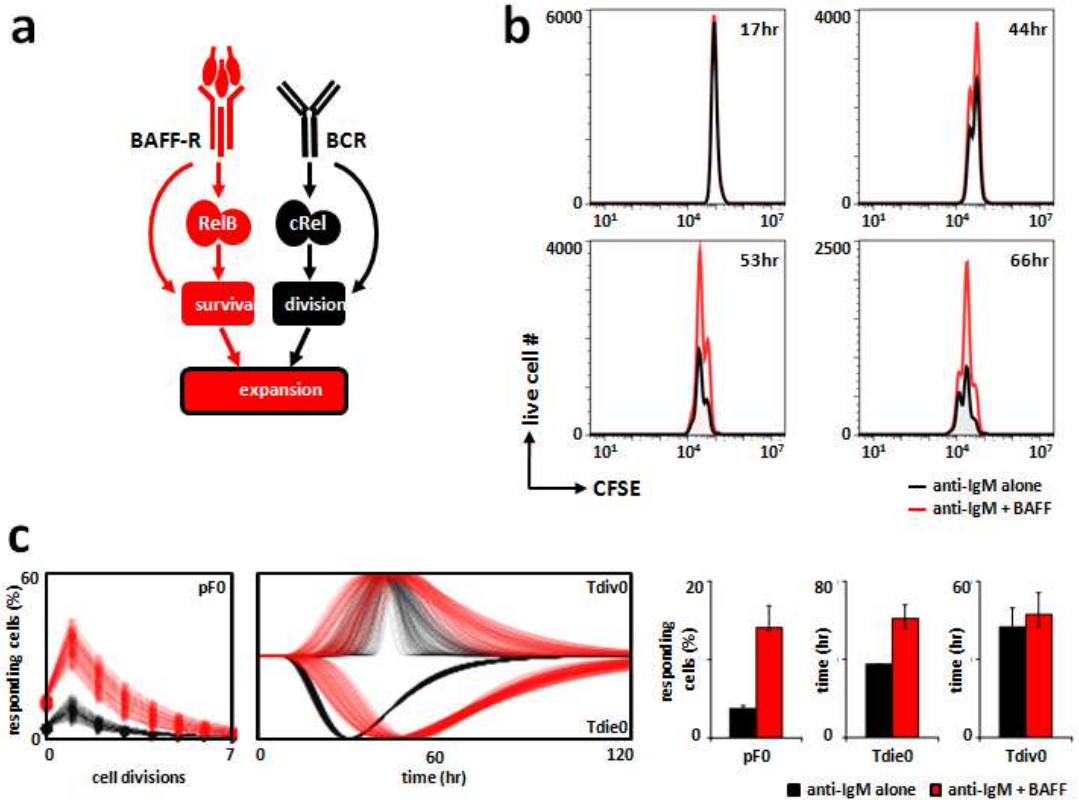

Figure 3.1. Proliferation and survival is enhanced in co-stimulated B cells.
(a) Schematic of the distinct canonical and non-canonical NFκB pathways identified in B cells. Antigenic stimulation leads to the release of cRel containing dimers into the nucleus, resulting in the activation of cell division programs. BAFF signals control non-canonical RelB dimer nuclear translocation, causing activation of survival and developmental profiles. Does cross-talk of these two critical receptors result in enhanced B cell expansion?
(b) In vitro proliferation of w*ild type* B cells labeled with CFSE and stimulated for three days with anti-IgM alone (black histogram) or anti-IgM + BAFF ligand (red histogram) and analyzed by flow cytometry. Live cell numbers gated by side- and forward-scatter profiles, followed by exclusion of 7AADHi population representing dead cells.
(c) The CFSE proliferation profiles of the wild type B cells stimulated with either anti-IgM or anti-IgM plus BAFF are analyzed using FlowMax running the Pcyton model to calculate the fraction of B cells responding to stimulation (pF0), the average time to division of undivided (Tdiv0) and average time to death of undivided (Tdie0) cells. Bar graphs used for summarizing the best fit cellular parameters along with the lognormal standard deviation.

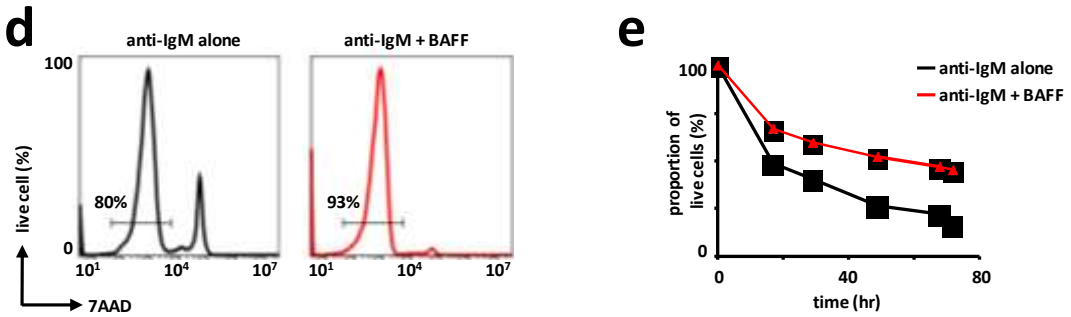

Figure 3.2. Survival is enhanced in BCR and BAFF-R co-stimulated B cells.
(d) Representative histograms (n = 3) of cell survival analyzed by 7AAD staining of B cells following 24 hours of stimulation by anti-IgM alone (black histogram), BAFF ligand alone (green), or anti-IgM + BAFF ligand (red histogram).
(e) Time course for viability of B cells stimulated with anti-IgM alone or anti-IgM + BAFF ligand. Live cells were identified by gating out $7AAD^{Hi}$ population. Data are means of triplicate cultures.

cRel enrichment in hyper-induced target genes

To characterize the transcriptomic response to co-stimulation we undertook RNA-seq analysis of splenic B cells stimulated with BAFF, IgM or both and an early (8 hr) and late (30 hr) timepoint. We found many more genes induced by IgM alone at the early than late timepoint (1339 vs. 978), but that co-stimulation resulted in more additional genes at the late than early timepoint (171 vs. 44) (Figure 3.3a). Similarly focusing on genes that are induced by IgM but show hyper-expression in co-simulation condition (defined as having an additional 2^0.5 fold increase in fold induction), we found more at the late than early time points (81 vs. 37), suggesting that co-stimulation primarily alters the late transcriptomic response. Interestingly, few of the previously identified mediators of B cell expansion are found among the set of hyperinduced genes (Figure 3.4b). In gene ontology analysis general terms such “metabolic process” or “biological regulation”/ “response to stimulus” score highly, whereas “regulation of cell death” or “cell division” do not (Figure 3.4c). However, what is remarkable that motif analysis of the regulatory region (-1000 to +300 of the transcription start site) of these hyperinduced genes reveals the κB motif as being substantially over-represented. In particular, using previously characterized NFκB binding motifs (Siggers et al., 2012) we find that the cRel:p50 binding motif as being present in 56% of the proximal regulatory regions of these hyper-expressed genes, regardless of their gene ontology categorization.

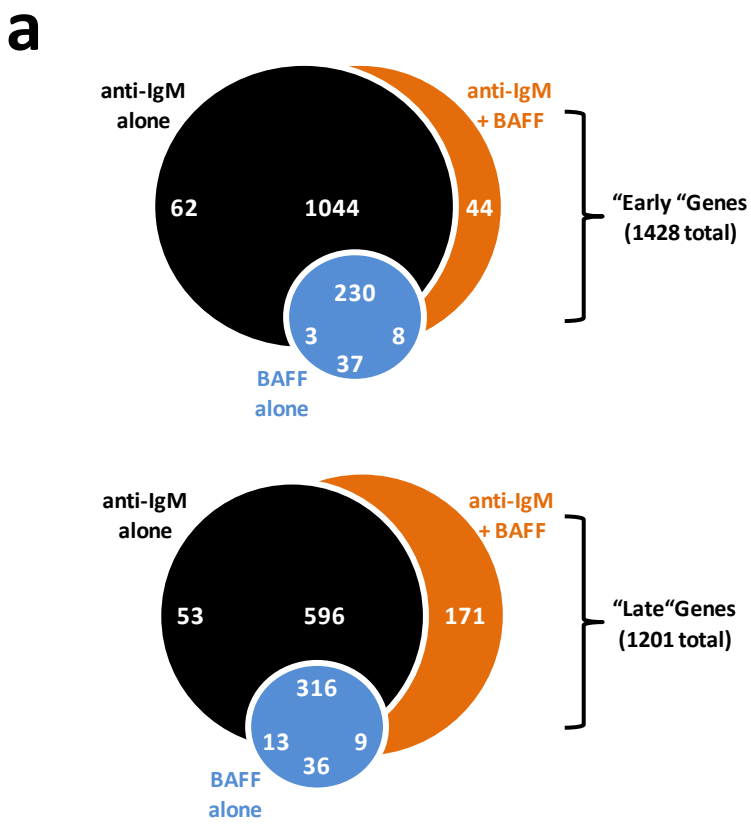

Figure 3.3. Hyper expressed genes in BCR and BAFF-R co-stimulated B cells possess high cRel enrichment.
(a) Venn diagram depicting genes which are induced > 2x fold over unstimulated naive wild type B cells (t = 0hr) in following eight and thirty hours of stimulation with 1) anti-IgM alone, 2) BAFF alone, or 3) anti-IgM and BAFF.
RNAseq analysis performed in collaboration with H. Birnbaum and J. Davis-Turak.

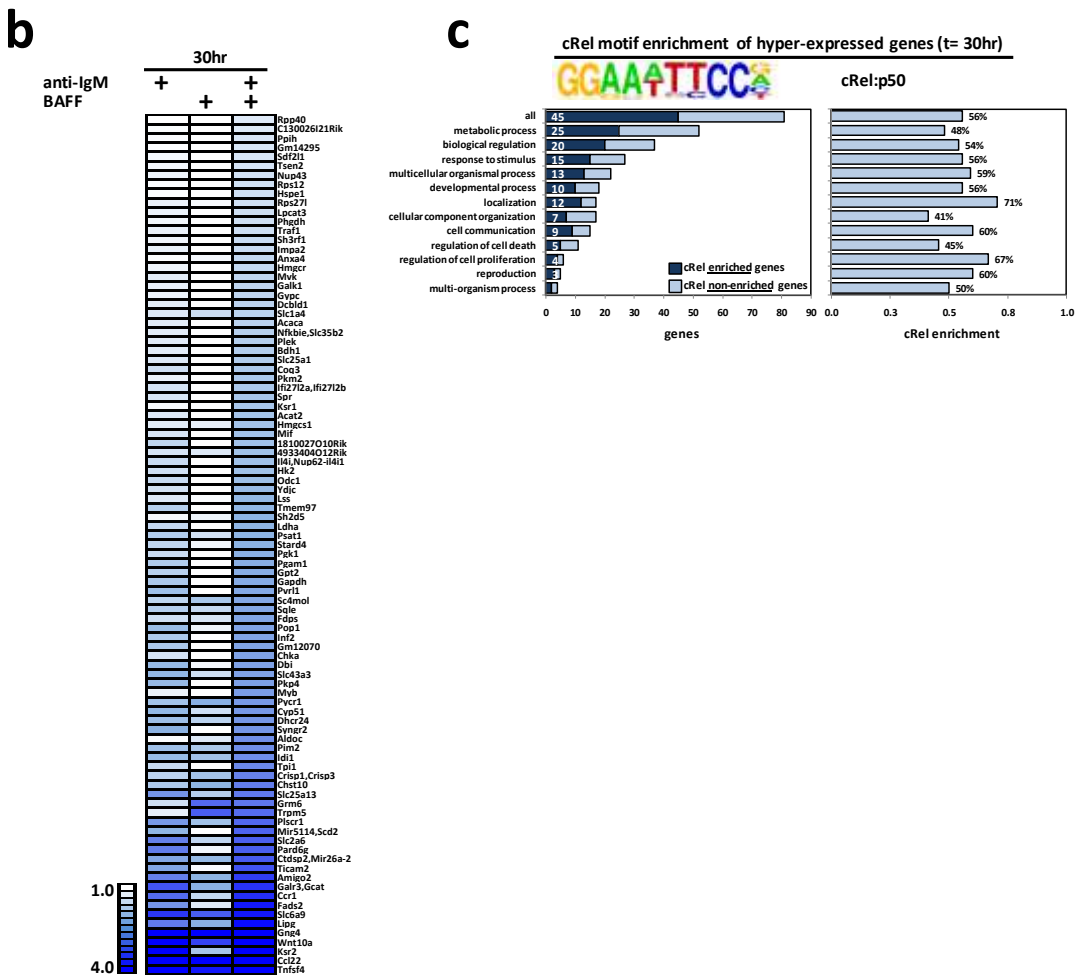

Figure 3.4. Hyper expressed co-stimulated genes contain motifs with high cRel enrichment.

(b)Heat map of "hyper" expressed genes. To be defines as hyper expressed, genes must first be induced >log2(0.5) over unstimulated naive wild type B cells (t = 0hr) in the single anti-IgM stimulated B cells at eight and thirty hours, than these genes must also have an additional log2(0.5) greater expression in the anti-IgM and BAFF co-stimulated B cells at the same time point.

(c) HOMER motif analysis for cRel enrichment of hyper expressed genes following thirty hours of stimulation with anti-IgM and BAFF. Genes are categorized into biological process also derived from HOMER.

RNAseq analysis performed in collaboration with H. Birnbaum and J. Davis-Turak.

Differential requirements for cRel and RelB

To determine the underlying molecular mechanisms of BAFF's function, we examined NFκB DNA binding activities biochemically using electrophoretic mobility shift assays coupled with antibodies to ablate all but the NFκB dimer of interest. Anti-IgM stimulation was found to induce a transient cRel activity within the first few hours, followed by a return to basal levels after 24 hours (Figure 3.5a). BAFF stimulation alone is also capable of activating cRel, with similar kinetics, but also results in long lasting RelB DNA binding activity. When both the BCR and BAFF-R are co-stimulated, we observed surprisingly little change in the induced RelB activity, but found that crel DNA binding activity was enhanced in amplitude at 12 hrs, as well as duration at 24 and 48 hr time points. Quantitation of the data shows that co-stimulation provides for "super-activated" cRel but not RelB (Figure 3.5a right panel).

We investigated the requirement of cRel and RelB NFκB family members in mediating BAFF's functions. First examining i*n vitro* survival of B cells cultured in BAFF, we found significant impairment in the absence of RelB (Figure 3.5b middle panel). Under identical conditions, we also note a decrease in the *crel*$^{-/-}$ B cells ability to respond to BAFF survival, although the phenotype was less severe (Figure 3.5b right panel). Charting a longer timecourse illustrates the importance of RelB (and to a lesser extent cRel) in BAFF-R induced survival of B cells *in vitro* (Figure 3.5c). Turning to BAFF's role as a co-stimulus during BCR-trigger B cell expansion, FACS analysis of

the CFSE labeled B cells revealed that enhanced B cell expansion in response to co-stimulation did not – surprisingly - require RelB (Figure 3.6d, top panel), but was dependent on cRel (Figure 3.6d bottom panel), just as B cell responses to BCR stimulation alone are known to be. Total cell number measurements reflect this requirement, as *crel*$^{-/-}$ B cells decline much more rapidly than wild type controls, and *relb*$^{-/-}$ show no obvious deficiency in these co-stimulation conditions (Figure 3.6e).Within 35 hours of stimulation with anti-IgM + BAFF, 43% and 42% of starting *wild type* and *relb*$^{-/-}$B cells were alive compared to only 9% of the *crel*$^{-/-}$ B cells. Computational deconvolution of the CFSE data confirmed that cRel is required not only for allowing B cells to enter the proliferative program (pFs) but also for cell survival (Tdie) (Figure 3.6f).

In sum, these studies indicate a differential requirement for cRel and RelB for BAFF's physiological function. When B cells are cultured in BAFF, its survival signals are mediated primarily by RelB; however, when BAFF functions as a co-stimulus to BCR, we observed super-activation of cRel which mediates the enhanced population expansion via increased proportions of cells entering the proliferative program.

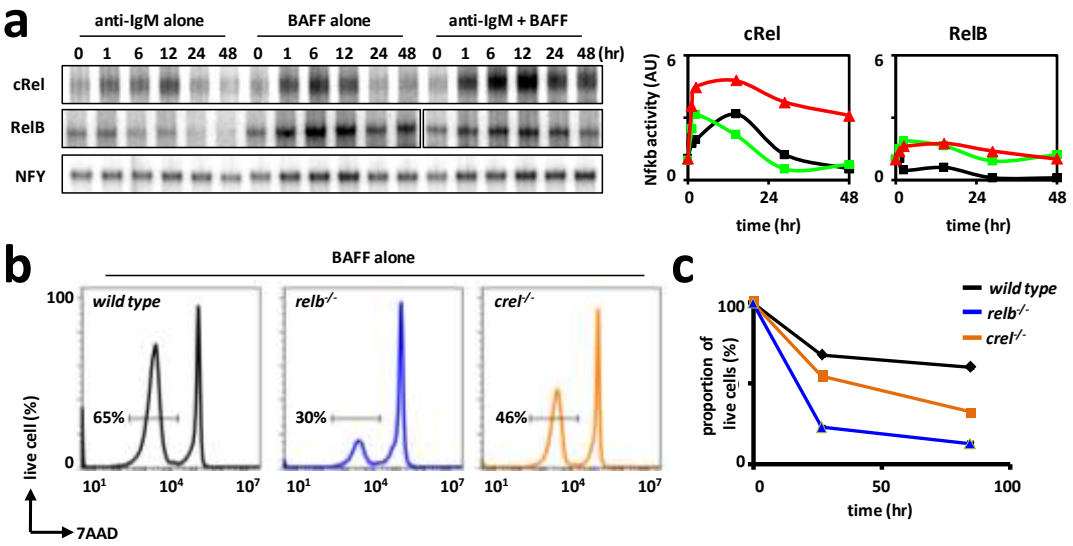

Figure 3.5. Co-stimulation of BCR and BAFF-R result in activation of cRel and RelB dimers, but enhanced expansion phenotype is dependent solely on cRel.
(a) Time course of NFκB cRel and RelB DNA binding activities monitored by EMSA. Nuclear extracts from wild-type B cells activated by indicated stimuli were collected and subjected to EMSA. NFY is used to ensure near-equal amounts of nuclear proteins were loaded onto gels. Signals were quantified and graphed relative to their respective resting cells (left).
(b) B cells derived for *wild type, crel, or* relb deficient mice were cultured with BAFF ligand for 76 hours, and the percentage of surviving B cells ($7AAD^-$) was assessed by FACS.
(c) Graphical representation of FACS plots examining B cell survival following three days of culture with BAFF ligand for *wild type, crel*$^{-/-}$ and *relb*$^{-/-}$B cells. Live cell numbers were obtained by exclusion of dead cells ($7AAD^{hi}$).

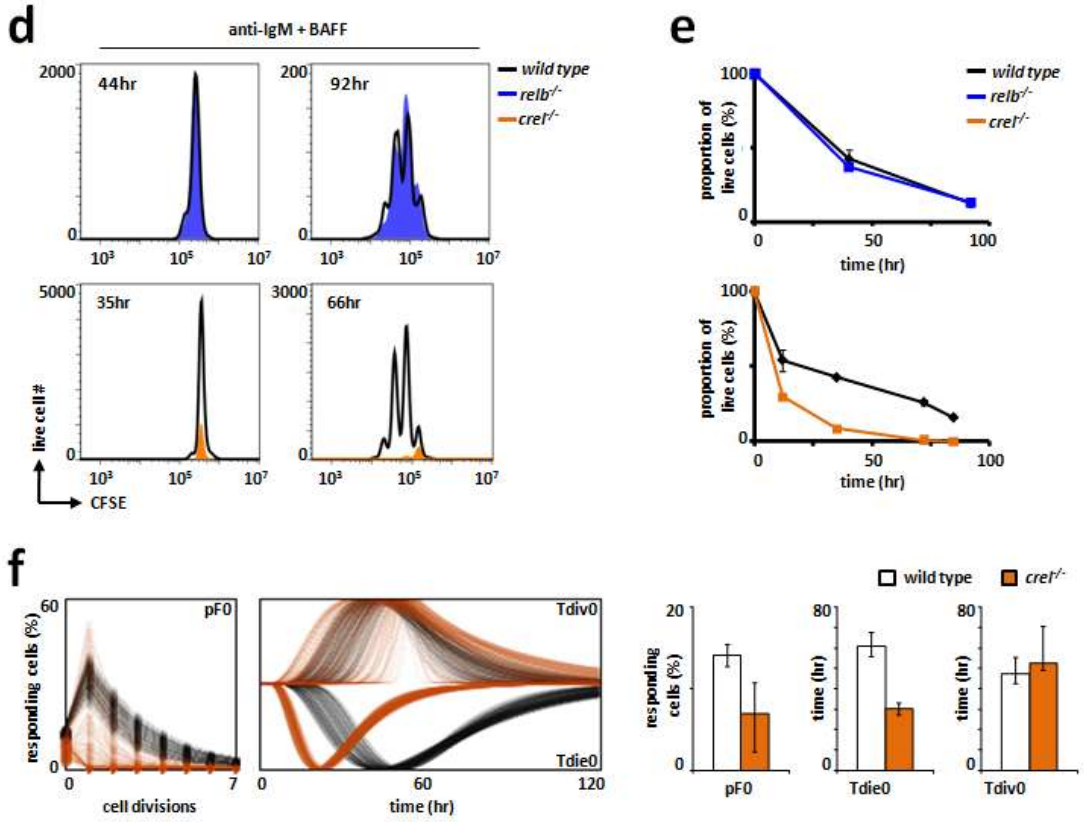

Figure 3.6. cRel, but not RelB is required for enhanced expansion phenotype observed in co-stimulated B cells.
(d) Wild type, RelB KO and cRel KO B cells are stained with CFSE, then stimulated with anti-IgM plus BAFF. Cell proliferation is examined by at the indicated time points by flow cytometry. Upper panel: w*ild type* (black histogram) versus *relb*$^{-/-}$(blue solid histogram). Lower panel: w*ild type* (black histogram) versus *crel*$^{-/-}$(orange solid histogram).
(e) Time course for viability of CFSE labeled *wild type, relb*$^{-/-}$*, and crel*$^{-/-}$B cells stimulated with anti-IgM + BAFF ligand. Live cells were identified by gating out 7AAD^{Hi} population. Fold survival calculated from unstimulated T=0hr controls.
(f) FlowMAX analysis of CFSE proliferation profiles for *wild type vs. crel*$^{-/-}$B cells from fig2d, lower panel. Bar graphs of pF0, Tdiv0, and Tdie0 with the lognormal standard deviation provided.

BAFF relieves IκBδ termination of cRel activity

To investigate the underlying molecular mechanism that allows BAFF to exert its physiological functions via distinct transcriptional effectors of the NFκB signaling system, we constructed a mathematical model of NFκB control in B cells in response to both canonical and non-canonical signals induced by BAFFR and BCR. The model was adapted from a previous version that recapitulates NFκB system control in murine embryonic fibroblasts (MEFs) in response to lymphotoxin-beta (LTβ) (Shih et al., 2012). A key mediator of signaling crosstalk is the NFκB responsive expression of p100 (Figure 3.7a), which not only functions as a substrate for p52 generation via processing (Basak et al., 2008; Stadanlick et al., 2008), but may also oligomerize to form the high molecular weight IκBsome, which presents a fraction of the C-terminal p100 ankyrin-repeat domain of p100 molecules as an IκB activity, termed IκBδ, capable of trapping latent NFκB dimers (Basak et al., 2007; Savinova et al., 2009). Thus cRel-driven p100 expression may not only potentiate RelB:p52 activation, but also lead to post-induction repression of cRel:p50, akin to IκBδ-mediated repression of LPS-induced NFκB activity in MEFs (Shih et al., 2009). BAFF as a co-stimulus with IgM might thus neutralize the IκBδ-mediated negative feedback loop.

To examine the basic premise of the model, we first examined stimulus-responsive IκB dynamics in B cells. The three canonical IκBs, IκBα, -β, -ε, showed characteristic degradation at early timepoint and induction at late

timepoints in response to anti-IgM, but this profile was unchanged when BAFF was applied as a co-stimulus (Figure 3.8). In contrast, p100 levels did not initially degrade but showed a huge induction beginning at 12hrs, and this induction was almost completely absent in co-stimulation conditions (Figure 3.7b) despite ample NFκB activity (Figure 3.5a) and nfkb2 mRNA synthesis (Figure 3.18b). Given p100's role in different complexes, we asked whether it was binding cRel. cRel immunoprecipitates revealed substantial p100 interaction following 24 hrs of IgM stimulation, but this was completely absent in the co-stimulation condition (Figure 3.7c). This time point coincides with the presence of a super-activated cRel species observed by EMSA (Figure 3.5a). In contrast, and as expected IκBα and IκBε showed interactions with cRel in both unstimulated and stimulated cells, with a transient reduction at the early timepoint. In sum, these observations support a model in which p100 forms IκBδ-containing complex, which limits cRel activity during BCR-triggered B cell expansion.

Development of the mathematical model began with careful delineation of the model's topology, leveraging previously established models. We may distinguish between reactions that control IκB synthesis and degradation (Figure 3.9a) and those that control NFκB monomer synthesis, dimerization, and interactions of dimers with IκBs (Figure 3.10a). The former represents an extension of the previously published four IκB model (Basak 2007, Shih 2009) and the latter is an extension of a model of RelA and RelB dimers in dendritic

cells (Shih et al., 2012) to include cRel as well. We thus account for all p50- and p52-containing dimers (8 or the 15), and the resulting 19 NFκB-IκB complexes. The two topologies are thus connected not only via protein-protein interactions but also via NFκB-dependent synthesis control, and the dual roles of nfkb2 in generating the NFκB dimerization partner p52 as well as an IκB family member. The aforementioned data contributed to an extensive training set described in the Supplementary information to achieve a B cell-specific parameterization. Simulations were produced with distributed kinase input profiles in recognition of the cell-to-cell heterogeneity of B cell responses; their average conforms to the measured profiles of IKK activity (Figure 3.11Figure 3.12). Likewise, averaged simulations of IκB and NFκB dynamics show that cRel-containing dimers are transiently induced peaking at about 5 hrs before returning to basal levels in response to BCR or BAFF-R stimulation alone (Figure 3.12d, 3rd row), whereas RelB-containing dimers are induced late and in a sustained and BAFF-R-dependent manner (Figure 3.12d, 4th row); these simulation outputs match our biochemical analysis (Figure 3.5a). Finally, simulation of the co-stimulation scenario (Figure 3.12d, 3rd column) provides for RelB and cRel with the observed superactivation of cRel at late times (20-40 hrs). The fact that early cRel activity is relatively unchanged suggests that augmented NEMO-IKK2 activity may not account for the observed superactivation. Instead, it coincides with a BAFF and NIK-dependent reduction in p100/IκBδabundance at late times, which neutralizes its BCR

driven expression. In sum, mathematical modeling demonstrates that kinetic considerations alone are sufficient to account for context-specific transcriptional effectors of BAFF-R signaling, without the need to invoke context-specific molecular mechanisms.

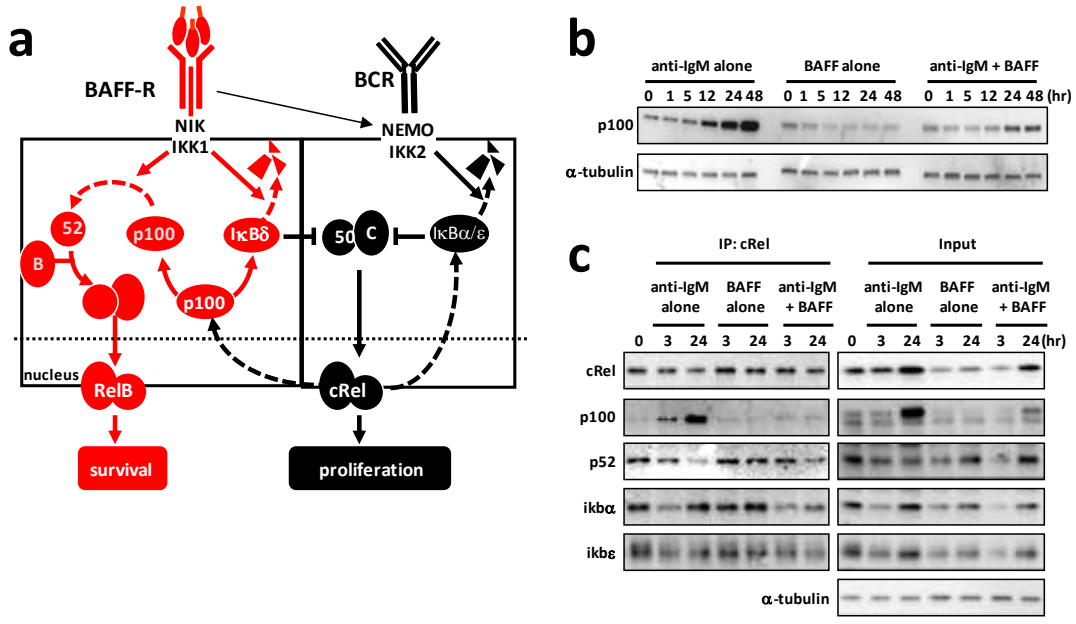

Figure 3.7. BAFF releases Iκbδ inhibition of cRel in BCR stimulated B cells.
(a) Proposed mechanism of Iκbδ inhibition of cRel in BCR stimulated B cells. In resting, naive state BAFF stimulation activates NIK:IKK1 kinase complex that results in p100 processing which allows for RelB:p52 nuclear translocation (fig2b). In an activated state, continuous BCR stimulation activates cRel dimers (fig 2a), resulting in the expression of the p100. This build of p100 substrate dimerizes to form the inhibitor Iκbδ, which binds cRel, acting as a brake on cRel activity. Alongside BAFF co-stimulation, the p100 build-up is prevented, allowing for persistent "super-activated" cRel.
(b) Cytoplasmic protein levels of p100 (Iκbδ) in stimulated wild type B cells were measured by western blot.
(c) Immunoblots of cRel coimmunoprecipitates prepared from wild-type B cells stimulated with anti-IgM, BAFF ligand, or anti-IgM and BAFF. Samples were normalized to cRel protein levels for comparison of kinetic IκBs-association to cRel under distinct stimuli conditions (left panel). Whole cell lysates (input) are also presented; α-tubulin serves as its loading control (right panel).

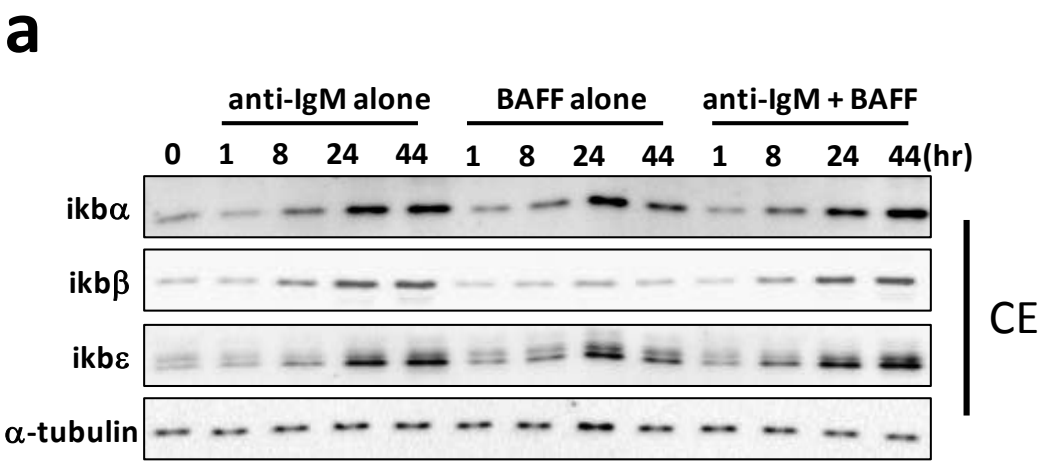

Figure 3.8. Canonical Iκbs protein levels in BCR stimulated B cells are unchanged in BCR and BAFF co-stimulated B cells.

(a) Cytoplasmic protein levels of Iκbα, Iκbβ, and Iκbε in stimulated *wild type* B cells were measured by western blot.

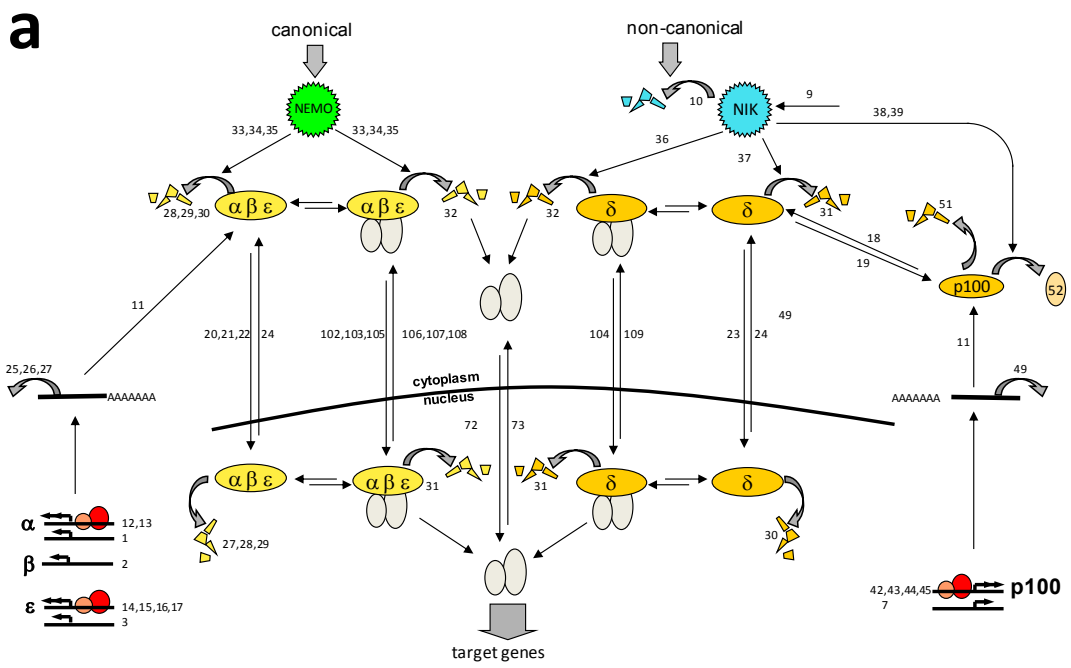

Figure 3.9. Diagram depicting the biochemical reactions of IκB synthesis and degradation that control NFκB signaling, as incorporated into the mathematical model.

(a)Parameter identifiers are found in Table 2.1, shown at the end of results section. Computational modeling performed by R. Tsui.

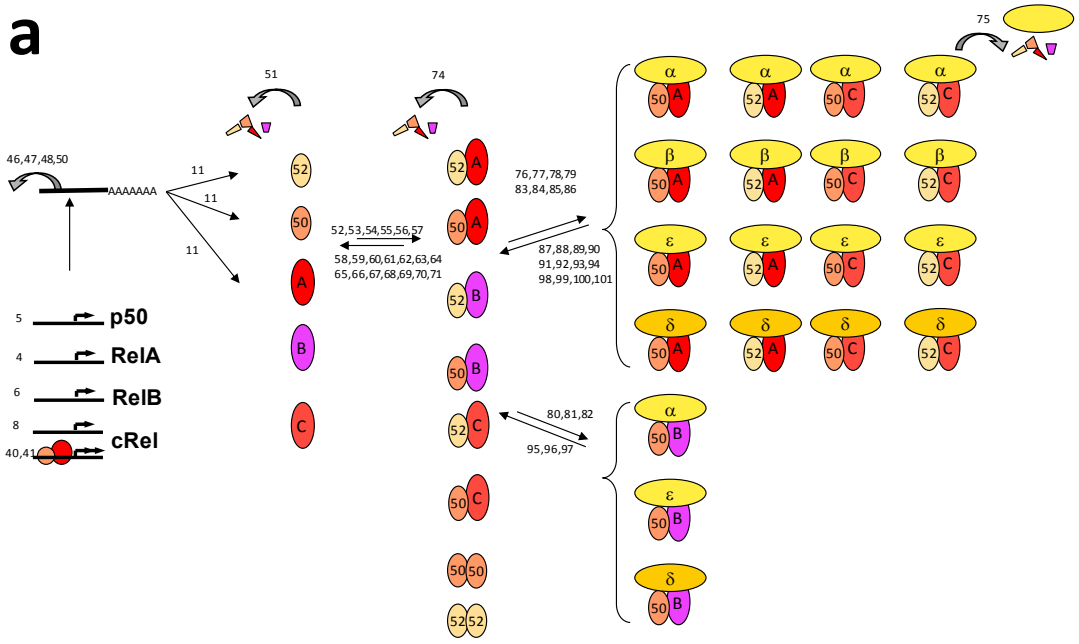

Figure 3.10. Diagram depicting biochemical reactions of NFκB synthesis and complex formation with NFκB inhibitors, as incorporated into the mathematical model.

(a)Parameter identifiers are found in Table 2.2, shown at the end of results section. Computational modeling performed by R. Tsui.

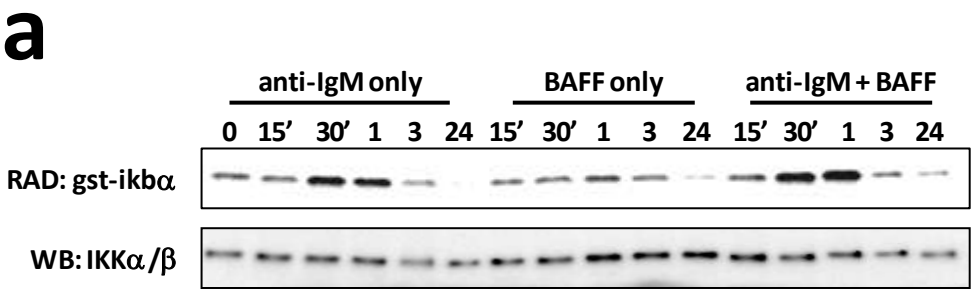

Figure 3.11. *In vitro* IKKγ kinase assay.
(a) NEMO-associated kinase activity was determined in *wild type* B cells in an in vitro IP-kinase assay upon stimulation with anti-IgM alone, 2) BAFF alone, or 3) anti-IgM and BAFF.

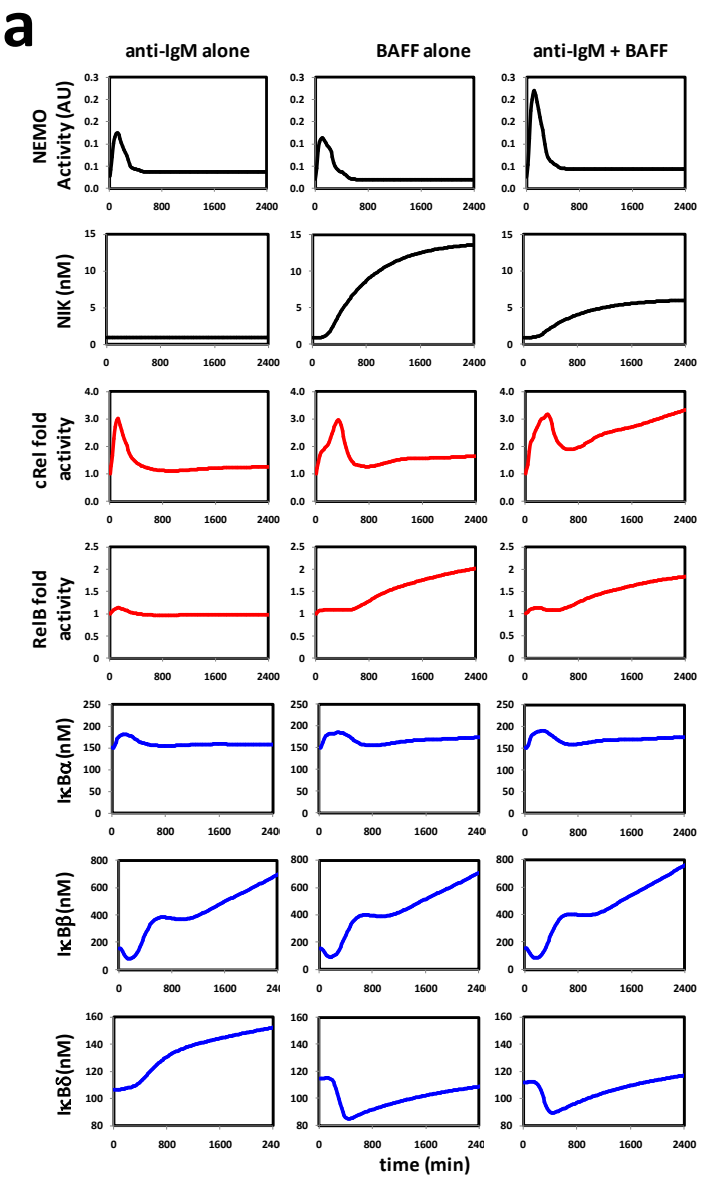

Figure 3.12. Mathematical model of NFκB dynamics in activated *wild type* B cells.

(a) Computational simulations are shown for the NEMO / IKK2-inducing stimuli anti-IgM (left column), NIK / IKK1-inducing survival signals mediated by BAFF ligand (middle column), and combined signals of anti-IgM plus BAFF ligand (left column). Simulation of NEMO / IKK2 and NIK /IKK1 activity profiles (top two panels, black), NFκB family members cRel, RelB, and p52 nuclear fold activities (middle three panels, red), and total cellular protein levels of IκBδ substrate, p100 (bottom panel, blue). Computational modeling performed by R. Tsui.

IκBδ limits cRel-mediated B cell expansion

The mathematical model encapsulates the key hypothesis that BAFF functions to relieve IκBδ-mediated termination of cRel activity. To examine this hypothesis further, we asked if a reduction in nfkb2 expression may be sufficient to phenocopy BAFF's co-stimulatory effect on B cell expansion. Mathematical modeling of a two-fold reduction in nfkb2 mRNA synthesis rate akin to nfkb2 heterozygosity, resulted in reduced p100 protein in both basal and IgM-stimulated condition in silico (Figure 3.13a) and in cells (Figure 3.13b-c), encouraging us to use the model to the experimental work. Importantly, we found that nfkb2$^{+/-}$ mice showed normal B cell populations (Figure 3.15a), unlike nfb2$^{-/-}$ mice, which show severe deficiencies in B cell development, in particular in the mature follicular B cell population, which are the primary responders to anti-IgM (Allman et al., 2001; Meyer-Bahlburg et al., 2008; Rawlings et al., 2012).

Using the model we predicted that the amount of cRel-p100 complex following 24 hours of anti-IgM stimulation would be reduced in *nfkb2*$^{+/-}$B cells compared to the *wild type* controls (Figure 3.13d). Quantitative co-immunoprecipitation experiments tracked well with the mathematical calculation (Figure 3.13e).Next, we simulated NFκB activation by anti-IgM; model simulations predicted that nfkb2 heterozygosity would result in enhanced cRel activity, showing a dramatic effect at late times (24-48 hrs), when cRel activity is usually terminated in wild type cells (Figure 3.13f).

Biochemical analysis confirmed this prediction, showing slight enhancement of peak activity, but dramatic failure to terminate cRel DNA binding activity at late times (Figure 3.13g). These studies establish the expression level of p100 as a remarkably sensitive parameter in terminating NFκB activity via the multimeric IκBδ.

To examine the cell biological consequence of enhanced cRel activation in *nfkb2*$^{+/-}$ B cells, we undertook CFSE dye dilution studies. FACS analysis revealed that anti-IgM stimulated *nfkb2*$^{+/-}$ B cells showed enhanced B cell expansion over *wild type* controls (Figure 3.14a). Computational deconvolution showed that this increased expansion was primarily due to increases in the fraction of cell entering the proliferative program (pF), slightly for generation zero (pF0) but more so for subsequent generations (pF2, 3, 4,etc). In contrast, cell cycle and survival times were not altered dramatically. Together, our data support the notion that IκBδ-containing IκBsome plays a key role in terminating antigen-triggered B-proliferative responses by controlling the duration of cRel transcription factor activity (Figure 3.14b).

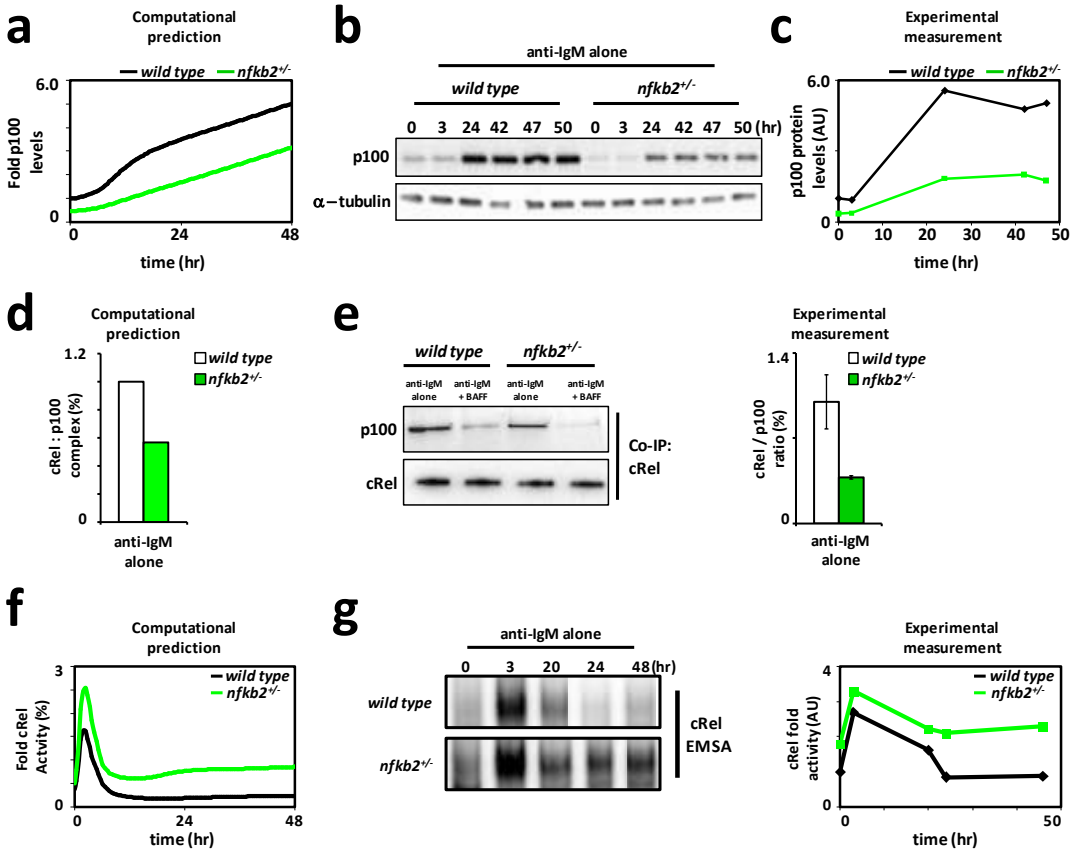

Figure 3.13. IκBδ limits the proliferative capacity of BCR-stimulated B cells.
(a) Model simulation of p100 protein levels in wild type and nfkb2 heterozygous B cells stimulated with anti-IgM alone.
(b) Immunoblot of p100 protein expression in *wild type* and *nfkb2*$^{+/-}$B cells stimulated with anti-IgM alone for two days.
(c) Signals from (b) were quantified and graphed relative to the wild type resting cells (0hr).
(d) Computational prediction of Iκbδ:cRel complex following 24 hours of anti-IgM stimulation in wild type (black bars) and nfkb2+/- (green bars) B cells.
(e) Immunoblots of cRel coimmunoprecipitates prepared from *wild type* and nfkb2 heterozygous B cells stimulated with anti-IgM or anti-IgM and BAFF for 24hours. Samples were normalized to cRel protein levels for comparison of kinetic IκBs-association to cRel under distinct stimuli conditions. Signals from immunoblot were quantified using Image Lab software and the association of cRel to p100 was graphed relative to the wild type stimulated B cells resting cells (24hr).
(f) Model simulation of the kinetics for active cRel in *wild type* and nfkb2 heterozygous B cells stimulated with anti-IgM alone.
(g) Representative cRel DNA binding activities in *wild type* and nfkb2 heterozygous B cells induced by anti-IgM alone were monitored by EMSA. Signals were quantified and plotted in the panel on right.
Computational modeling performed by R. Tsui.

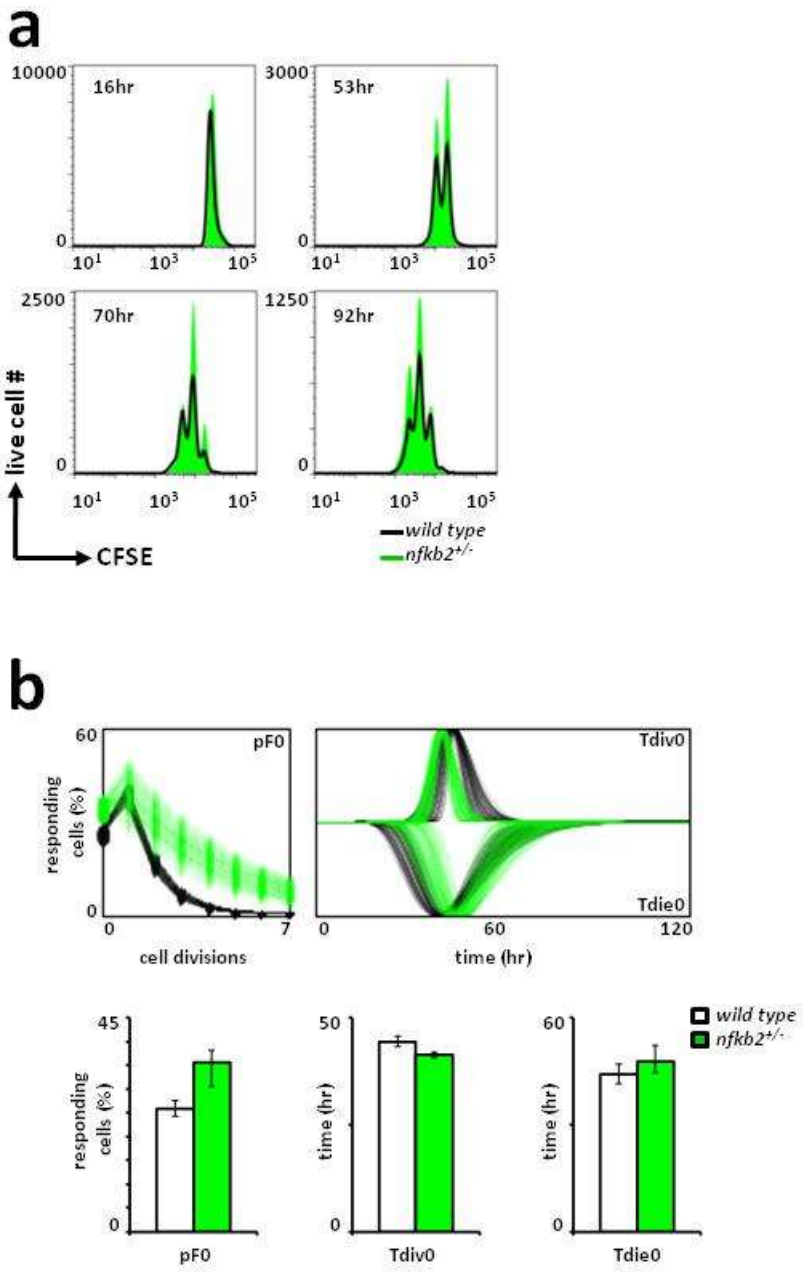

Figure 3.14. Consequence of lowering nfkb2 expression levels enhanced B cell expansion with single BCR stimulation.
(a) Time course of B cell proliferation for *wild type* (black histogram) and *nfkb2*$^{+/-}$ (green histogram) B cells labeled with CFSE and 7AAD.
(b) FlowMax analysis of CFSE proliferation assay in (h) of *wild type* (black) and *nfkb2*$^{+/-}$(green) B cells.

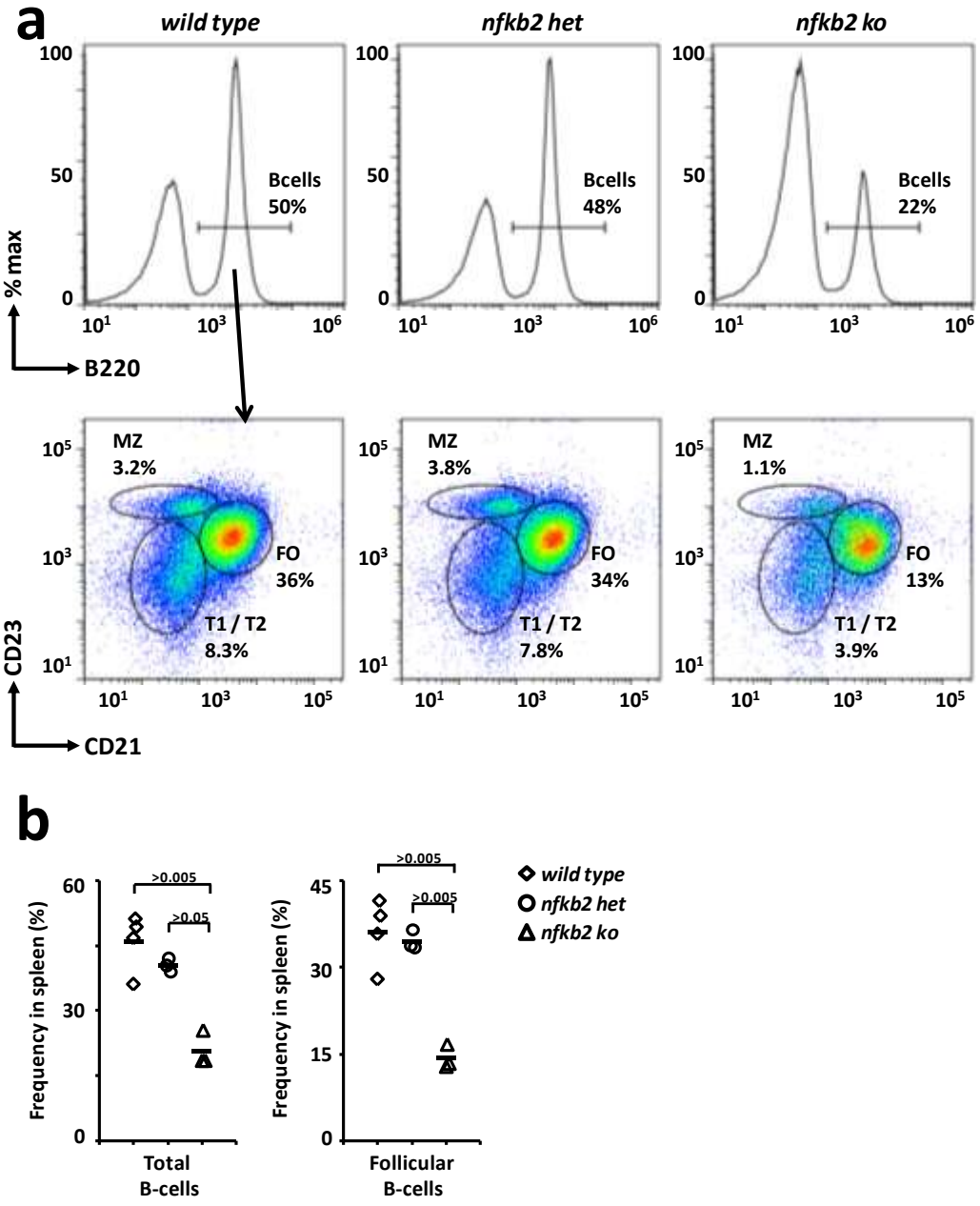

Figure 3.15. B cell maturation is defective in nfkb2 knockout mice, but unaffected in the heterozygous state.
(a) Splenocytes were stained with anti-B220, anti-CD21, and anti-CD23. B cells populations are gated as $B220^{+}$. For cells gated on $B220^{+}$, marginal zone B cells ($CD21^{hi}CD23^{lo}$), follicular B cells ($CD21^{lo}CD23^{hi}$)and transitional 1 and transitional 2 B cells ($CD21^{lo}CD23^{lo}$) are shown. Representative data of 4 mice shown.
(b) The numbers of total and FO B cells obtained from (A) are displayed graphically.

Nfkb1/p105 stabilizes IκBδ to control B cell expansion

To further test the model that the IκBδ-containing IκBsome is critical in terminating cRel-driven B cell proliferative responses, we sought ways to perturb IκBsome formation. Previous biochemical studies described an assembly pathway for high-molecular weight IκBsome involving mutually stabilizing interactions between the nfkb1 and nfkb2 gene products p105 and p100 (Savinova et al., 2009). In addition, p50 and p52 have been described to compete for a limited pool of activation domain-containing NFκB proteins, RelA, cRel and RelB (Basak et al., 2008). Both mechanisms determine the balance of IκBsome formation and processing to p50/p52.These interdependencies in p100 and p105 control (diagramed in Figure 3.16a) allow for compensation for the loss of p50 by p52 in MEFs (Hoffmann et al., 2003) but also result in the loss of p100 in *nfkb1*$^{-/-}$ MEFs as well as lymphotoxin-beta responsiveness (Basak et al., 2008) and lymph node formation (Lo et al., 2006).

We examined whether nfkb1 might be required for efficient IκBδ function. To this end, we first investigated the possibility that nfkb2 transcription may be perturbed in *nfkb1*$^{-/-}$B cells. However, our quantitative analysis indicated that the steady-state and inducible levels of *nfκb2* mRNA are similar in *nfkb1*$^{-/-}$ and wild type knockout B cells (Figure 3.18a). However, immunoblot analysis of *nfkb1*$^{-/-}$ B cell extracts indicated lower levels of p100 protein and elevated levels of p52 (Figure 3.16b). This decrease in the

available p100 pool was also evident in the cytoplasmic fraction (Figure 3.16c). Immunoblotting nuclear fractions for cRel, we found a greater cRel availability in BCR stimulated *nfkb1*$^{-/-}$ B cells than wild type counterparts (Figure 3.16d). These data suggest that nfkb1-deficiency destabilizes p100-containing IκBδ to neutralize its inhibition of cRel activity. As expected, coimmunoprecipitation analysis confirmed that p100 was bound to cRel in a stimulus responsiveness fashion in *wild type* B cells, but to a dramatically lower extent in *nfkb1*$^{-/-}$ B cells (Figure 3.17e).

We next examined BCR-triggered B cell expansion using CFSE dye dilution analysis. FACS analysis revealed enhanced proliferative responses in *nfkb1*$^{-/-}$ B cells compared to wild type controls (Figure 3.17f). Computational deconvolution identified that the phenotype was primarily due to a greater fraction of cells entering the proliferative program with only minor changes in cell division or survival times (Figure 3.17g). Although nfkb1/p50 is a known dimerization partner of cRel, our data suggests that nfkb1's role in antigen-responsive B cell expansion is primarily to limit the proliferative program by stabilizing IκBδ.

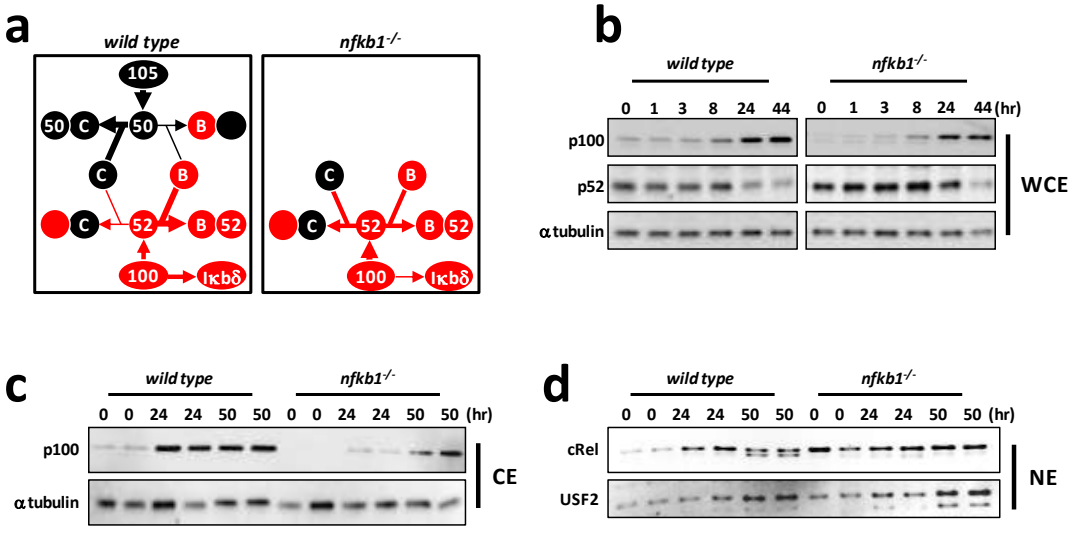

Figure 3.16. nfkb1/p105 limits the proliferative capacity of B cells by stabilizing IκBδ.
(a) Proposed schematic of Nfkb1 and Nfkb2 protein fates in *wild type* and *nfkb1*$^{-/-}$B cells. In normal wild type B cells, p105 and p100 mainly functions in the NFkB signaling system as precursors of p50 and p52. p105 is processed into p50 and dimerizes predominantly with cRel and RelA, and to a lesser extent RelB. p100 processing yields p52, which in turn dimerizes with RelB primarily. A second function of p100 is to form the inhibitory molecule Iκbδ (left panel). In nfkb1 knockout B cells, p105 / p50 absence leaves cRel and RelA molecules without their primary binding partner. This in turn shifts the available pool of p100 to be processed into p52 in order overcome the loss of p50; as a result less p100 is accessible to form Iκbδ (right panel).
(b) Immunoblots for wild type and nfkb1 knockout whole cell extracts (WCE) were used to examine p52 protein levels under BCR stimulation. α-tubulin was used to ensure equal loading for all samples.
(c) Comparison of cytoplasmic p100 protein expression in *wild type* and *nfkb1*$^{-/-}$B cells which have been stimulated with anti-IgM alone by immunoblotting.
(d) Western blot of nuclear cRel for wild type and nfkb1 KO B cells stimulated with anti-IgM alone. Nuclear marker USF2 is used as a loading control loading.

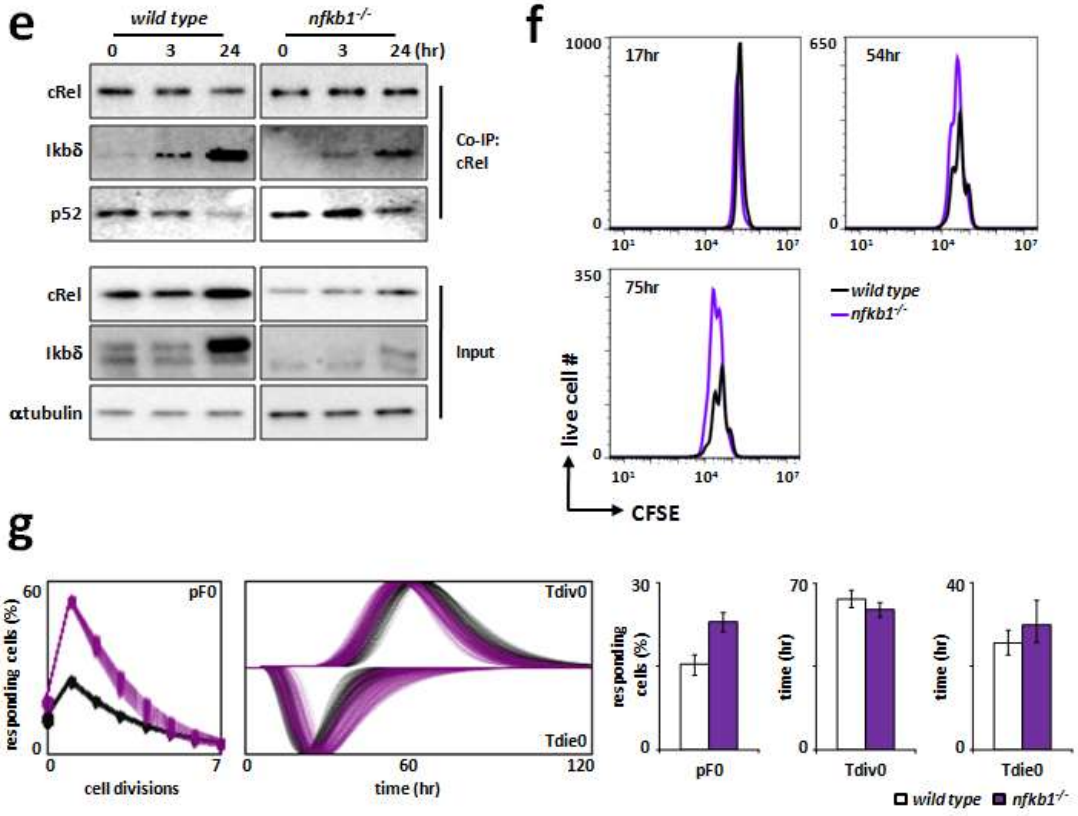

Figure 3.17. Disruption of Iκbsome formation by deletion of nfkb1 leads to enhanced B cell expansion with single BCR stimulation.
(e) Western blots of immunoprecipitates to monitor IκBδ (and p52?) associated with cRel during BCR stimulation time course. cRel was immunoprecipitated from B cell whole cell extracts and prepared at indicated time points. Whole cell lysates (input) are also shown; α-tubulin serves as its loading control
(f) FACS analysis for *in vitro* proliferation of w*ild type* (black histogram) and *nfkb1*$^{-/-}$ (purple histogram) B cells labeled with CFSE and stimulated for three days with anti-IgM alone. Live cell numbers gated by side- and forward-scatter profiles, followed by exclusion of 7AAD^{Hi} population representing dead cells
(g) FlowMAX analysis of CFSE proliferation profiles for *wild type* (black) vs. *nfkb1*$^{-/-}$ (purple)B cells from (f).

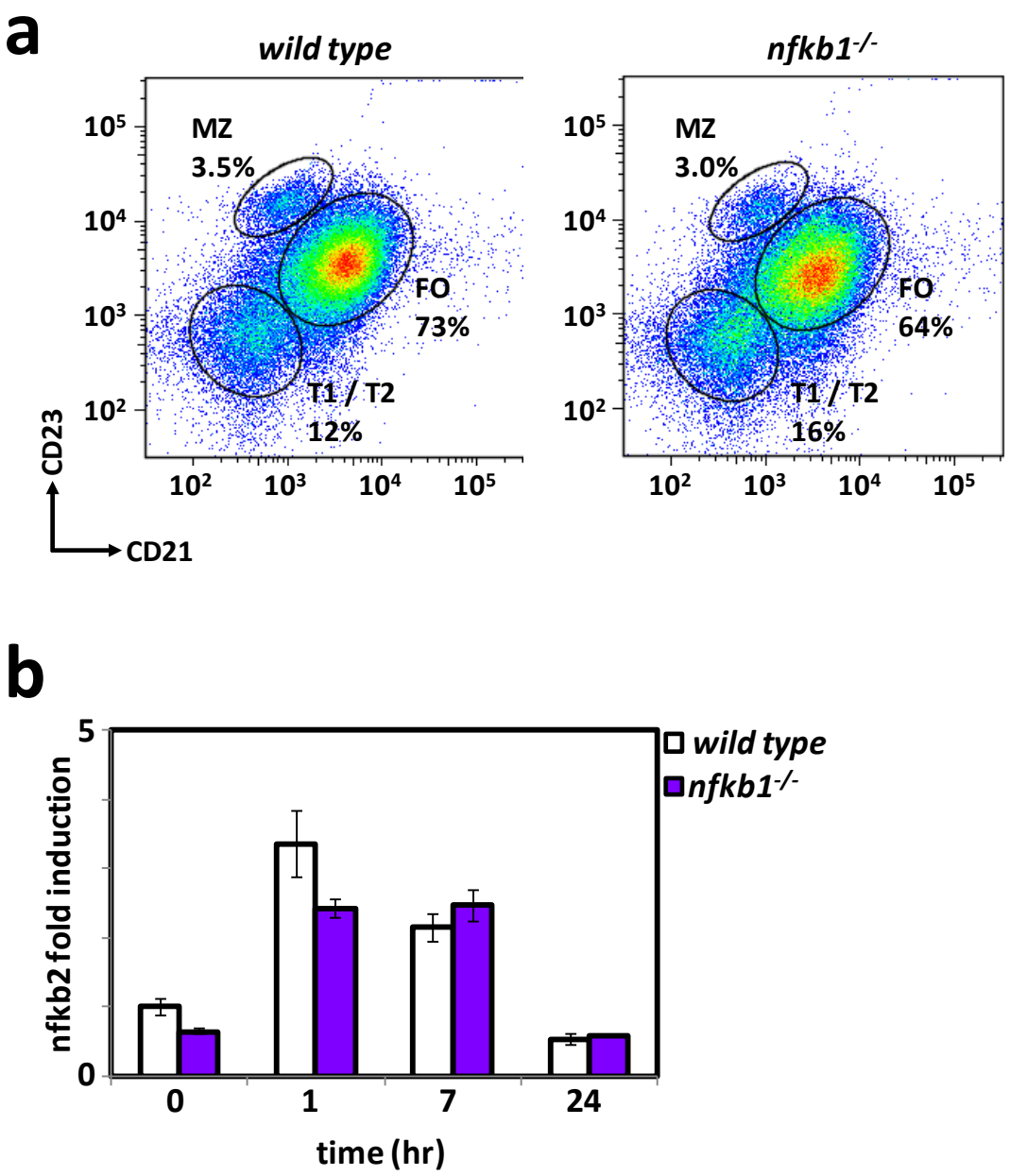

Figure 3.18. Normal B cell development is observed in nfkb1 deficient mice.
(a) Splenocytes were stained with anti-B220, anti-CD21, and anti-CD23. B cells populations are gated as $B220^{+}$ (not shown). For cells gated on $B220^{+}$, marginal zone B cells ($CD21^{hi}CD23^{lo}$), follicular B cells ($CD21^{lo}CD23^{hi}$)and transitional 1 and transitional 2 B cells ($CD21^{lo}CD23^{lo}$) are shown. Representative data of 4 mice shown.
(b) Nfkb2 gene expression was monitored by qPCR in *wild type* and *nfkb1*$^{-/-}$B cells stimulated with anti-IgM.

Table 3.1. Parameter table (reaction rates) for mathematical model (see Figure 3.9).
Computational modeling performed by R. Tsui.

Reaction	Location	Param No.	Parameter Value	Category	Source
Reaction rates determined by transcriptional programs and cytokine levels					
=> tIκBα (basal)	Nucleus	1	4.8e-3 nM/min	IκB Synth.	Parameter value chosen to fit mRNA and protein Expression profiles as measure by RNase Protection (RPA) and Western blot assays, reformulated from Werner et al. (2008) to fit a Hill function
=> tIκBβ (basal)	Nucleus	2	1.2e-3 nM/min	IκB Synth.	*Refer to #1*
=> tIκBε (basal)	Nucleus	3	1.2e-4 nM/min	IκB Synth.	*Refer to #1*
=> tRelA (basal)	Nucleus	4	3.6e-5 nM/min	NFκB Synth.	*Refer to #1*
=> tp50 (basal)	Nucleus	5	2.9e-5 nM/min	NFκB Synth.	*Refer to #1*
=> tRelB (basal)	Nucleus	6	4.2e-5 nM/min	NFκB Synth.	*Refer to #1/*Fitted
=> tp100 (basal)	Nucleus	7	8e-7 nM/min	NFκB Synth.	*Refer to #1*
=> cRel (basal)	Nucleus	8	3.6e-6 nM/min	NFκB Synth.	*Refer to #1/*Fitted
=> NIK	Cytoplasm	9	4.2e-2 nM/min	NIK Synth.	Set to yield measured abundance in conjunction with #10
NIK =>	Cytoplasm	10	4.6e-2	NIK Deg.	Based on estimated 15-minute half-life/ Fitted
IκB Reactions					
mRNA => mRNA + protein	Nuc - > Cyt	11	12 proteins/ mRNA/min	Translation	Derived from the elongation rate of the ribosome and corrected for the nucleotide spacing between adjacent ribosomes on the same transcript 1800 nt min^{-1} / 150 nt = 12 min^{-1}
=> tIκBα (A50/A52-induced)	Nucleus	12	200 Fold over constitutive	IκB Synth.	*Refer to #1*
Hill K_d (A50/A52-induced)	Nucleus	13	150 nM	IκB Synth.	*Refer to #1*
=> tIκBε (A50/A52-induced, 37 min. delay)	Nucleus	14	25 Fold over constitutive	IκB Synth.	*Refer to #1*
Hill K_d (A50/A52-induced)	Nucleus	15	150 nM	IκB Synth.	*Refer to #1*
=> tIκBε (C50/C52-induced, 37 min. delay)	Nucleus	16	250 Fold over constitutive	IκB Synth.	*Refer to #1*
Hill K_d (C50/C52-induced)	Nucleus	17	150 nM	IκB Synth.	*Refer to #1*
p100 + p100 => IκBδ	Cyt, Nuc	18	1.2e-2 nM^{-1} min^{-1}	IκB Synth.	Estimated a $K_d$of 10nM
IκBδ => p100 + p100	Cyt, Nuc	19	1.2e-2 min^{-1}	IκB Synth.	*Refer to #19*
IκBα(c) => IκBα(n)	Cyt -> Nuc	20	6.0e-2 min^{-1}	Transport	Adapted from (Shih et al., 2009)
IκBβ(c) => IκBβ(n)	Cyt -> Nuc	21	9.0-3 min^{-1}	Transport	(Shih et al., 2009)
IκBε(c) => IκBε(n)	Cyt -> Nuc	22	4.5e-2 min^{-1}	Transport	(Shih et al., 2009)
IκBδ(c) => IκBδ(n)	Cyt -> Nuc	23	4.5e-2 min^{-1}	Transport	(Shih et al., 2009)

Table 3.2. Parameter table (IκB reactions) for mathematical model.

IκB[α/β/ε/δ](n) => IκB[α/β/ε/δ](c)	Nuc - > Cyt	24	1.2e-2 min^{-1}	Transport	(Shih et al., 2009)
tIκBα =>	Nucleus	25	2.9e-2 min^{-1}	IκB Deg.	mRNA half-life measurements using actinomycin-D treatment of cells and RPA (unpublished results)
tIκBβ =>	Nucleus	26	2.9e-3 min^{-1}	IκB Deg.	*Refer to #25*
tIκBε =>	Nucleus	27	3.8e-3 min^{-1}	IκB Deg.	*Refer to #25*
IκBα =>	Cyt, Nuc	28	0.12 min^{-1}	IκB Deg.	(Shih et al., 2009)
IκBβ =>	Cyt, Nuc	29	0.12 min^{-1}	IκB Deg.	(Shih et al., 2009)
IκBε =>	Cyt, Nuc	30	1.2e-2 min^{-1}	IκB Deg.	Based on estimated 1 hour half-life
IκBδ =>	Cyt, Nuc	31	3e-3 min^{-1}	IκB Deg.	Based on estimated 4 hour half-life
IκB[α/β/ε/δ]-NFκB => NFκB	Cyt, Nuc	32	2.4e-4 min^{-1}	IκB Deg.	Based on estimated 48 hour half-life
IκBα => (NEMO-mediated)	Cytoplasm	33	1.4e-3 nM^{-1} min^{-1}	IκB Deg.	Based on measured IκB degradation time courses given numerical input curves
IκBαNFκB => NFκB (NEMO-mediated)	Cytoplasm	33	1.4e-3 nM^{-1} min^{-1}	IκB Deg.	
IκBβ => (NEMO-mediated)	Cytoplasm	34	4.5e-4 nM^{-1} min^{-1}	IκB Deg.	*Refer to # 33*
IκBβNFκB => NFκB (NEMO-mediated)	Cytoplasm	34	4.5e-4 nM^{-1} min^{-1}	IκB Deg.	*Refer to # 33*
IκBε => (NEMO-mediated)	Cytoplasm	35	3.4e-4 nM^{-1} min^{-1}	IκB Deg.	*Refer to # 33*
IκBεNFκB => NFκB (NEMO-mediated)	Cytoplasm	35	3.4e-4 nM^{-1} min^{-1}	IκB Deg.	*Refer to # 33*
IκBδ => (NIK-mediated)	Cytoplasm	36	0.6 nM^{-1} min^{-1}	IκB Deg.	V_{max} and K_m of NIK-mediated reactions based on protein degradation and estimated NIK abundances.
IκBδNFκB => NFκB (NIK-mediated)	Cytoplasm	36	0.6 nM^{-1} min^{-1}	IκB Deg.	
IκBδ => (NIK-mediated, K_m)	Cytoplasm	37	100 nM	IκB Deg.	*Refer to #36*
NF-κB reactions					
p100 => p52 (NIK-mediated)	Cytoplasm	38	5.0e-2 nM^{-1} min^{-1}	NFκB Synth.	*Refer to #36*
p100 => p52 (NIK-mediated, p100 K_m)	Cytoplasm	39	10 nM	NFκB Synth.	*Refer to #36*
=> cRel (A50/A52/C50/C52-induced, 1 hr delay)	Nucleus	40	200 Fold over constitutive	NFκB Synth.	*Refer to #1/Fitted*

Table 3.3. Parameter table (NFκB reactions) for mathematical model.

Hill K_d (A50/A52/C50/C52-induced)	Nucleus	41	150 nM	NFκB Synth.	*Refer to #1/Fitted*
=> tp100 (A50/A52-induced, 4 hr delay)	Nucleus	42	1000 Fold over constitutive	NFκB Synth.	*Refer to #1/Fitted*
Hill K_d (A50/A52-induced)	Nucleus	43	50 nM	NFκB Synth.	*Refer to #1/Fitted*
=> tp100 (C50/C52-induced, 4 hr delay)	Nucleus	44	1500 Fold over constitutive	NFκB Synth.	*Refer to #1/Fitted*
Hill K_d (C50/C52-induced)	Nucleus	45	50 nM	NFκB Synth.	*Refer to #1/Fitted*
tRelA =>	Nucleus	46	2.9e-3 min^{-1}	NFκB Deg.	*Refer to #25*
tp50 =>	Nucleus	47	2.9e-3 min^{-1}	NFκB Deg.	*Refer to #25*
tRelB =>	Nucleus	48	2.9e-3 min^{-1}	NFκB Deg.	*Refer to #25*
tp100 =>	Nucleus	49	9.6e-4 min^{-1}	NFκB Deg.	*Refer to #25*
tcRel=>	Nucleus	50	9.6e-4 min^{-1}	NFκB Deg.	*Refer to #25*
RelA =>	Cyt, Nuc	51	2.3e-2 min^{-1}	NFκB Deg.	Based on estimated 0.5 hour half-life of NF-κB monomers
p50 =>	Cyt, Nuc	51	2.3e-2 min^{-1}	NFκB Deg.	
RelB =>	Cyt, Nuc	51	2.3e-2 min^{-1}	NFκB Deg.	
p100 =>	Cyt, Nuc	51	2.3e-2 min^{-1}	NFκB Deg.	
cRel =>	Cyt, Nuc	51	2.3e-2 min^{-1}	NFκB Deg.	
p52 =>	Cyt, Nuc	51	2.3e-2 min^{-1}	NFκB Deg.	
RelA + p50 => RelAp50	Cyt, Nuc	52	1.9e-3 nM^{-1} min^{-1}	NFκB Synth.	Based on dimerization studies (unpublished results)
RelA + p52 => RelAp52	Cyt, Nuc	52	1.9e-3 nM^{-1} min^{-1}	NFκB Synth.	
RelB + p52 => RelBp52	Cyt, Nuc	53	9.6e-4 nM^{-1} min^{-1}	NFκB Synth.	
RelB + p50 => RelBp50	Cyt, Nuc	53	3e-4 nM^{-1} min^{-1}	NFκB Synth.	
cRel + p50 => cRelp50	Cyt, Nuc	54	9.6e-4 nM^{-1} min^{-1}	NFκB Synth.	
cRel + p52 => cRelp52	Cyt, Nuc	55	1.9e-3 nM^{-1} min^{-1}	NFκB Synth.	
p50 + p50 => p50p50	Cyt, Nuc	56	1.8e-3 nM^{-1} min^{-1}	NFκB Synth.	
p52+ p52 => p52p52	Cyt, Nuc	57	1.8e-3 nM^{-1} min^{-1}	NFκB Synth.	
RelAp50 => RelA + p50	Cyt	58	1.9e-2 min^{-1}	NFκB Synth.	Based on dimerization studies (unpublished results)
RelAp52 => RelA + p52	Cyt	59	3.8e-2min^{-1}	NFκB Synth.	
RelBp52 => RelB + p52	Cyt	60	1.4e-2 min^{-1}	NFκB Synth.	
RelBp50 => RelB + p50	Cyt	61	4.6e-3 min^{-1}	NFκB Synth.	
cRelp50 => cRel + p50	Cyt	62	1.4e-3 min^{-1}	NFκB Synth.	
cRelp52 => cRel + p52	Cyt	63	1.4e-3min^{-1}	NFκB Synth.	

Table 3.4. Parameter table (additional NFκB reaction) for mathematical model.

RelAp50 => RelA + p50	Nuc	64	1.9e-3 min^{-1}	NFκB Synth.	Estimated 10 fold higher affinity due to DNA binding
RelAp52 => RelA + p52	Nuc	65	3.8e-3min^{-1}	NFκB Synth.	*Refer to #64*
RelBp52 => RelB + p52	Nuc	66	1.4e-3 min^{-1}	NFκB Synth.	*Refer to #64*
RelBp50 => RelB + p50	Nuc	67	4.6e-3 min^{-1}	NFκB Synth.	*Refer to #64*
cRelp50 => cRel + p50	Nuc	68	1.4e-4 min^{-1}	NFκB Synth.	*Refer to #64*
cRelp52 => cRel + p52	Nuc	69	1.4e-4min^{-1}	NFκB Synth.	*Refer to #64*
p50p50 => p50 + p50	Cyt, Nuc	70	5.4e-2min^{-1}	NFκB Synth.	Based on dimerization studies (unpublished results)
p52p52 => p52+ p52	Cyt, Nuc	71	5.4e-2min^{-1}	NFκB Synth.	Based on dimerization studies (unpublished results)
RelAp50(c) => RelAp50(n)	Cyt -> Nuc	72	5.4 min^{-1}	Transport	(Shih et al., 2009)
RelAp52(c) => RelAp52(n)	Cyt -> Nuc	72	5.4 min^{-1}	Transport	(Shih et al., 2009)
RelBp52(c) => RelBp52(n)	Cyt -> Nuc	72	5.4 min^{-1}	Transport	(Shih et al., 2009)
RelBp50(c) => RelBp50(n)	Cyt -> Nuc	72	5.4 min^{-1}	Transport	(Shih et al., 2009)
cRelp50(c) => cRelp50(n)	Cyt -> Nuc	72	5.4 min^{-1}	Transport	(Shih et al., 2009)
cRelp52(c) => cRelp52(n)	Cyt -> Nuc	72	5.4 min^{-1}	Transport	(Shih et al., 2009)
p50p50(c) => p50p50(n)	Cyt -> Nuc	72	5.4 min^{-1}	Transport	(Shih et al., 2009)
p52p52(c) => p52p52(n)	Cyt -> Nuc	72	5.4 min^{-1}	Transport	(Shih et al., 2009)
NFκB(n) => NFκB(c)	Nuc -> Cyt	73	4.8e-3 min^{-1}	Transport	(Shih et al., 2009)
RelAp50 =>	Cyt, Nuc	74	2.4e-4 min^{-1}	NFκB Deg.	Based on estimated 48 hour half-life
RelAp52 =>	Cyt, Nuc	74	2.4e-4 min^{-1}	NFκB Deg.	
RelBp50 =>	Cyt, Nuc	74	2.4e-4 min^{-1}	NFκB Deg.	
RelBp52 =>	Cyt, Nuc	74	2.4e-4 min^{-1}	NFκB Deg.	
cRelp50 =>	Cyt, Nuc	74	2.4e-4 min^{-1}	NFκB Deg.	
cRelp52 =>	Cyt, Nuc	74	2.4e-4 min^{-1}	NFκB Deg.	
p50p50 =>	Cyt, Nuc	74	2.4e-4 min^{-1}	NFκB Deg.	
p52p52 =>	Cyt, Nuc	74	2.4e-4 min^{-1}	NFκB Deg.	
IκB[α/β/ε/δ]-NFκB => IκB[α/β/ε/δ]	Cyt, Nuc	75	2.4e-4 min^{-1}	NFκB Deg.	*Refer to #74*
IκB:NF-κB interactions					
IκBα + RelA:p50 => IκBα:RelA:p50	Cyt, Nuc	76	3e-3 nM^{-1} min^{-1}	IκB-NFκB interaction	Adapted from *Alves et. al 2013*

Table 3.5. Parameter table (IκB:NFκB interaction) for mathematical model.

IκBβ + RelA:p50 => IκBβ:RelA:p50	Cyt, Nuc	77	2e-4 nM^{-1} min^{-1}	IκB-NFκB interaction	Adapted from *Alves et. al 2013*
IκBε + RelA:p50 => IκBε:RelA:p50	Cyt, Nuc	78	1.3e-3 nM^{-1} min^{-1}	IκB-NFκB interaction	Adapted from *Alves et. al 2013*
IκBδ + RelA:p50 => IκBδ:RelA:p50	Cyt, Nuc	79	6e-4 nM^{-1} min^{-1}	IκB-NFκB interaction	Adapted from *Alves et. al 2013*
IκBα + RelA:p52 => IκBα:RelA:p52	Cyt, Nuc	76	3e-3 nM^{-1} min^{-1}	IκB-NFκB interaction	Adapted from *Alves et. al 2013*
IκBβ + RelA:p52 => IκBβ:RelA:p52	Cyt, Nuc	77	2e-4 nM^{-1} min^{-1}	IκB-NFκB interaction	Adapted from *Alves et. al 2013*
IκBε + RelA:p52 => IκBε:RelA:p52	Cyt, Nuc	78	1.3e-3 nM^{-1} min^{-1}	IκB-NFκB interaction	Adapted from *Alves et. al 2013*
IκBδ + RelA:p52 => IκBδ:RelA:p52	Cyt, Nuc	79	6e-4 nM^{-1} min^{-1}	IκB-NFκB interaction	Adapted from *Alves et. al 2013*
IκBα + RelB:p50 => IκBα:RelB:p50	Cyt, Nuc	80	1.3e-3 nM^{-1} min^{-1}	IκB-NFκB interaction	Adapted from *Alves et. al 2013*
IκBε + RelB:p50 => IκBε:RelB:p50	Cyt, Nuc	81	1.3e-3 nM^{-1} min^{-1}	IκB-NFκB interaction	Adapted from *Alves et. al 2013*
IκBδ + RelB:p50 => IκBδ:RelB:p50	Cyt, Nuc	82	6e-4 nM^{-1} min^{-1}	IκB-NFκB interaction	Adapted from *Alves et. al 2013*
IκBα + cRel:p50 => IκBα:cRel:p50	Cyt, Nuc	83	3e-3 nM^{-1} min^{-1}	IκB-NFκB interaction	Adapted from *Alves et. al 2013*
IκBβ +cRel:p50 => IκBβ:cRel:p50	Cyt, Nuc	84	2.1e-4 nM^{-1} min^{-1}	IκB-NFκB interaction	Adapted from *Alves et. al 2013*
IκBε + cRel:p50 => IκBε:cRel:p50	Cyt, Nuc	85	1.3e-3 nM^{-1} min^{-1}	IκB-NFκB interaction	Adapted from *Alves et. al 2013*
IκBδ + cRel:p50 => IκBδ:cRel:p50	Cyt, Nuc	86	1.98e-2 nM^{-1} min^{-1}	IκB-NFκB interaction	Adapted from *Alves et. al 2013*
IκBα + cRel:p52 => IκBα:RelA:p52	Cyt, Nuc	83	3e-3 nM^{-1} min^{-1}	IκB-NFκB interaction	Adapted from *Alves et. al 2013*
IκBβ + cRel:p52 => IκBβ:cRel:p52	Cyt, Nuc	84	2.1e-4 nM^{-1} min^{-1}	IκB-NFκB interaction	Adapted from *Alves et. al 2013*
IκBε + cRelp52 => IκBε:cRel:p52	Cyt, Nuc	85	1.3e-3 nM^{-1} min^{-1}	IκB-NFκB interaction	Adapted from *Alves et. al 2013*
IκBδ + cRel:p52 => IκBδ:cRel:p52	Cyt, Nuc	86	1.98e-2 nM^{-1} min^{-1}	IκB-NFκB interaction	Adapted from *Alves et. al 2013*
IκBα:RelA:p50 => IκBα + RelA:p50	Cyt, Nuc	87	6e-4 min^{-1}	IκB-NFκB interaction	Fitted (dependent on 76-79)
IκBβ:RelA:p50 => IκBβ + RelA:p50	Cyt, Nuc	88	1.7e-2 min^{-1}	IκB-NFκB interaction	
IκBε:RelA:p50 => IκBε + RelA:p50	Cyt, Nuc	89	6e-3 min^{-1}	IκB-NFκB interaction	

Table 3.6. Parameter table (NFκB:IκB interactions) for mathematical model.

IκBα:RelA:p52 => IκBα + RelA:p52	Cyt, Nuc	91	6e-4 min^{-1}	IκB-NFκB interaction	Fitted (dependent on 76-79)
IκBβ:RelA:p52 => IκBβ + RelA:p52	Cyt, Nuc	92	1.7e-2 min^{-1}	IκB-NFκB interaction	
IκBε:RelA:p52 => IκBε + RelA:p52	Cyt, Nuc	93	6e-3 min^{-1}	IκB-NFκB interaction	
IκBδ:RelA:p52 => IκBδ + RelA:p52	Cyt, Nuc	94	8.4e-4 min^{-1}	IκB-NFκB interaction	
IκBα:RelB:p50 => IκBα + RelB:p50	Cyt, Nuc	95	3e-2 min^{-1}	IκB-NFκB interaction	Fitted (dependent on 80 -82)
IκBε:RelB:p50 => IκBε + RelB:p50	Cyt, Nuc	96	3e-2 min^{-1}	IκB-NFκB interaction	
IκBδ:RelB:p50 => IκBδ + RelB:p50	Cyt, Nuc	97	8.4e-4 min^{-1}	IκB-NFκB interaction	
IκBα:cRel:p50 => IκBα + cRel:p50	Cyt, Nuc	98	4.8e-3 min^{-1}	IκB-NFκB interaction	Fitted (dependent on 83-86)
IκBβ:cRel:p50 => IκBβ +cRel:p50	Cyt, Nuc	99	1.7e-2 min^{-1}	IκB-NFκB interaction	
IκBε:cRel:p50 => IκBε + cRel:p50	Cyt, Nuc	100	2.7e-5 min^{-1}	IκB-NFκB interaction	
IκBδ:cRel:p50 => IκBδ + cRel:p50	Cyt, Nuc	101	8.4e-4 min^{-1}	IκB-NFκB interaction	
IκBα:cRel:p52 => IκBα + cRel:p52	Cyt, Nuc	98	4.8e-3 min^{-1}	IκB-NFκB interaction	Fitted (dependent on 83-86)
IκBβ:cRel:p52 => IκBβ + cRel:p52	Cyt, Nuc	99	1.7e-2 min^{-1}	IκB-NFκB interaction	
IκBε:cRel:p52 => IκBε + cRelp52	Cyt, Nuc	100	2.7e-5 min^{-1}	IκB-NFκB interaction	
IκBδ:cRel:p52 => IκBδ + cRel:p52	Cyt, Nuc	101	8.4e-4 min^{-1}	IκB-NFκB interaction	
IκBα:NFκB(c) => IκBα:NFκB(n)	Cyt -> Nuc	102	0.28 min^{-1}	Transport	(Shih et al., 2009)
IκBβ:NFκB(c) => IκBβ:NFκB(n)	Cyt -> Nuc	103	0.028 min^{-1}	Transport	(Shih et al., 2009)
IκBδ:NFκB(c) => IκBδ:NFκB(n)	Cyt -> Nuc	104	0.028 min^{-1}	Transport	Based on slower import rate of IκBβ:NFκB
IκBε:NFκB(c) => IκBε:NFκB(n)	Cyt -> Nuc	105	0.14 min^{-1}	Transport	(Shih et al., 2009)
IκBα:NFκB(n) => IκBα:NFκB(c)	Nuc -> Cyt	106	0.84 min^{-1}	Transport	(Shih et al., 2009)
IκBβ:NFκB(n) => IκBβ:NFκB(c)	Nuc -> Cyt	107	0.42 min^{-1}	Transport	(Shih et al., 2009)
IκBε:NFκB(n) => IκBε:NFκB(c)	Nuc -> Cyt	108	0.42 min^{-1}	Transport	(Shih et al., 2009)
IκBδ:NFκB(n) => IκBδ:NFκB(c)	Nuc -> Cyt	109	0.42 min^{-1}	Transport	(Shih et al., 2009)

Table 3.7. Species table for mathematical model (see Figure 3.10)
Computational modeling performed by R. Tsui.

	Species	Location
1	tIκBα	Nucleus
2	tIκBβ	Nucleus
3	tIκBε	Nucleus
4	tRelA	Nucleus
5	tp50	Nucleus
6	tp100	Nucleus
7	tcRel	Nucleus
8,9	IκBα	Nucleus, Cytoplasm
10,11	IκBβ	Nucleus, Cytoplasm
12,13	IκBε	Nucleus, Cytoplasm
14,15	IκBδ	Nucleus, Cytoplasm
16,17	RelA	Nucleus, Cytoplasm
18,19	RelB	Nucleus, Cytoplasm
20,21	cRel	Nucleus, Cytoplasm
22,23	p50	Nucleus, Cytoplasm
24,25	p100	Nucleus, Cytoplasm
26,27	p52	Nucleus, Cytoplasm
28,29	RelAp50	Nucleus, Cytoplasm
30,31	RelAp52	Nucleus, Cytoplasm
32,33	RelBp50	Nucleus, Cytoplasm
34,35	RelBp52	Nucleus, Cytoplasm
36,37	cRelp50	Nucleus, Cytoplasm
38,39	cRelp52	Nucleus, Cytoplasm
40,41	p50p50	Nucleus, Cytoplasm
42,43	p52p52	Nucleus, Cytoplasm
44,45	IκBα:RelAp50	Nucleus, Cytoplasm
46,47	IκBβ:RelAp50	Nucleus, Cytoplasm
48,49	IκBε:RelAp50	Nucleus, Cytoplasm
50,51	IκBδ:RelAp50	Nucleus, Cytoplasm
52,53	IκBα:RelAp52	Nucleus, Cytoplasm
54,55	IκBβ:RelAp52	Nucleus, Cytoplasm
56,57	IκBε:RelAp52	Nucleus, Cytoplasm
58,59	IκBδ:RelAp52	Nucleus, Cytoplasm
60,61	IκBα:RelBp50	Nucleus, Cytoplasm
62,63	IκBε:RelBp50	Nucleus, Cytoplasm
64,65	IκBδ:RelBp50	Nucleus, Cytoplasm
66,67	IκBα:cRelp50	Nucleus, Cytoplasm
68,69	IκBβ:cRelp50	Nucleus, Cytoplasm
70,71	IκBε:cRelp50	Nucleus, Cytoplasm
72,73	IκBδ:cRelp50	Nucleus, Cytoplasm
74,75	IκBα:cRelp52	Nucleus, Cytoplasm
76,77	IκBβ:cRelp52	Nucleus, Cytoplasm
78,79	IκBε:cRelp52	Nucleus, Cytoplasm
80,81	IκBδ:cRelp52	Nucleus, Cytoplasm
82	NEMO	Cytoplasm
83	NIK	Cytoplasm

Discussion

The TNF-superfamily member BAFF has diverse and critical roles during B-lymphocyte development, homeostasis and pathogen-responsive expansion. Using an iterative systems biology approach we identified two distinct molecular mechanisms and transcriptional effectors that mediate BAFF's biological functions. Whereas BAFF's survival function in maturing B cells is mediated by the well-known NFκB non-canonical pathway effector RelB, its function as a co-stimulus in antigen-responsive B cell expansion is mediated by super-activation of cRel to enhance the fraction of cells entering the proliferative program. Our studies reveal that a key determinant of the duration of the cRel-driven proliferative program is the p105/p100-containing IκBsome, which mediates IκBδ activity. BAFF activation of the non-canonical pathway neutralizes this potent negative feedback loop.

Previous studies established that BAFF is a potent activator of the non-canonical NFκB signaling pathway, which activates NFκB activity via NEMO-independent kinase complexes involving IKK1 and NIK. A hallmark of non-canonical NFκB activity is the activation of RelB:p52, which relies on IKK1-dependent processing of nfkb2/p100. However, the appearance of this dimer is also dependent on NEMO-dependent canonical NFκB activity, as both p100 and RelB expression are regulated by RelA and cRel (Basak et al., 2008; Muller and Siebenlist, 2003;Shinners et al., 2007). Interestingly, although NIK, cRel and RelA have been shown to be required for normal B cell homeostasis,

no genetic evidence points to the necessity of RelB. Here we show that BAFF-induced survival of resting splenocytes is indeed RelB-dependent. However, BAFF's function as a co-stimulus during antigen-receptor triggered B cell expansion was RelB-independent prompting us to search for other relevant effectors. In stark contrast, cRel was shown to be vital for BCR and BAFF induced proliferation.

Under the control of antigenic stimulation, cRel is required for progression from G1 to S-phase (Grumont et al., 1998). Expression of E2F3, a transcription factor involved in G1/S phase progression (Humbert et al., 2000) was shown to be impaired in BCR activated cRel-deficient B cells (Cheng et al., 2003). Apoptosis typically increases following B cell activation, eliminating B cells that are incapable of undergoing normal cell division (Gerondakis and Siebenlist, 2010). Viability of BCR-activated B cells is reliant on cRel directly inducing the transcription of genes encoding the Bcl-2 pro-survival homologs, A1/Blf1 (Grumont et al., 1999) and Bcl-xL (Cheng et al., 2003). Therefore, within the context of BCR triggered expansion, cRel is the decisive NFκB effector given its importance to cell cycle and pro-survival gene expression programs. Indeed, transcriptomic studies identified genes with enhanced expression during co-stimulation that revealed a cRel binding motif in associated regulatory regions.

Despite RelB's importance to BAFF-mediated survival *in vitro,* it showed negligible effect on co-stimulated B cell expansion, suggesting that

BAFF survival signals do not play a significant role in the enhance B cell expansion phenotype. With the enormity of cRel to BCR controlled proliferation, along with biochemical analysis of persistent cRel present in antigenic stimulated B cells following the addition of BAFF-triggered NIK activity lead us to that a examine the negative feedback on cRel activity. Our lab previously reported activation of lymphotoxin-β, like BAFF-R a member of the TNF superfamliy, can prompt IKK1-dependent inactivation of a fourth IkB, termed Iκbδ, allowing for activation of the RelA:p50 dimer (Basak et al., 2007). We have also established that IκBδ can act as a negative feedback regulator of RelA in inflammatory signaling (Shih et al., 2009). Indeed, in the presence of BAFF, BCR-stimulated B cells failed to restrict cRel activity to a transient keep, allowing for larger numbers of cells entering the proliferative program, particularly at later generations.

We further established our Iκbδ model experimentally through 1) attenuation of p100 expression levels (with nfkb2 heterozygous B cells) and 2) disruption of IκBsome assembly (using nfkb1 deficient B cells) would relieve single BCR stimulated B cells of cRel inhibition and experience enhanced expansion mimicking the co-stimulated state. Finally, with the construction of a B cell specific mathematical model, we showed the IκBδ mechanism is sufficient to recapitulate cRel activation dynamics in B cells.

Collectively, proposed a model (Figure 3.19) by which the BAFF-R synergizes with the BCR to hyperactivate cRel, resulting in enhanced B cell

expansion. BCR activates NEMO-dependent kinase IKK, (and to a lesser extent BAFF-R as shown in chapter 2) resulting in activation of latent cRel-containing dimers prompting proliferative gene expression programs (Castro et al., 2009; Cheng et al., 2003; Feng et al., 2004; Grumont et al., 1998). Persistent antigenic signals lead to the expression of p100 (Stadanlick et al., 2008)(Castro et al., 2009), which in turn dimerize into the inhibitory complex Iκbδ, inhibiting cRel driven cell proliferation. BAFF-R activation involves NIK-IKK1 complex, elicits processing of p100 to p52 and generation of the RelB:p52 transcription factor controlling cell survival and maturation (Clise-Dwyer et al., 2001; Hsu et al., 2002; Rahman and Manser, 2004; Sasaki et al., 2004).The addition of BAFF-R signals with antigenic functions, lead to NIK activity preventing accumulation of p100, allowing cRel activity to continue unperturbed.

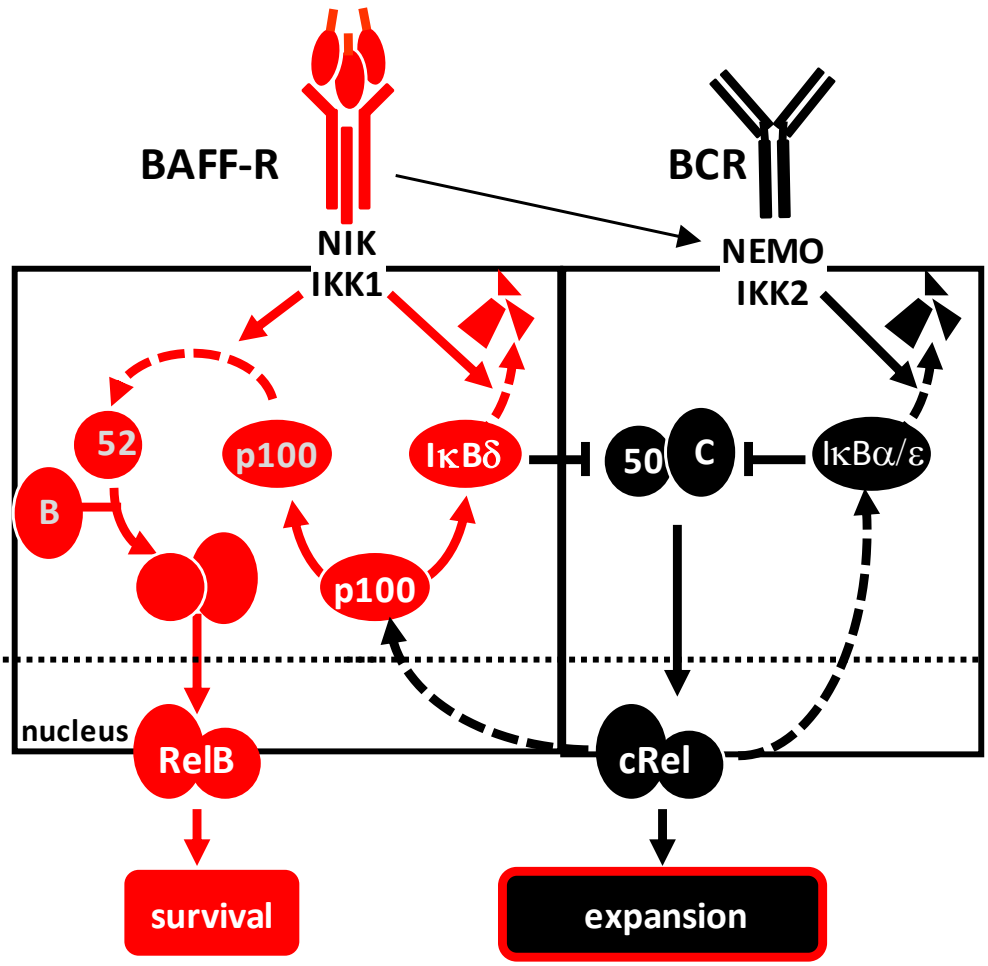

Figure 3.19 Inhibitory feedback of IκBδ on BCR triggered cRel activity is relieved with additional BAFF-R signals.
In normal resting B cells, tonic BCR signals allow for expression of cRel. While insufficient for proliferation, cRel induces expression of p100 (nfkb2), which in turn is processed to p52 via NIK / IKK1 induced BAFF-R activity. p52 then dimerizes with RelB and translocates to the nucleus to turn on BAFF-mediated survival gene expression.
In activated B cell setting, BCR recognition of foreign antigen activates cRel cell cycle and survival programs in addition to p100 protein expression. The build-up of p100 protein results in dimerization of p100 into IκBδ, which shuts off BCR / cRel driven B cell expansion. This formation of p100 into Iκbδ is prevented with the addition of BAFF induces NIK-IKK1 activity, which allows for persistent cRel activation and continued expression of pro-expansion genes.

Materials and Methods

Cell isolation and culture. Spleens were harvested from C57Bl6 mice *wild type* (Jackson Labs, Bar Harbor, MN). The collected spleens were homogenized using frosted glass slide grinding. For B cell isolation, homogenized splenocytes were incubated with anti-CD43 (Ly-48) microbeads for 15 minutes at room temperature. Following this incubation, the cells were washed with Hanks Buffered salt solution (HBSS) (Gibco 14170) containing 1% FCS, 10mM HEPES (Gibco 15630) and 1% FCS (Sigma F2442) and separated over a magnetic column (LS column MiltenyiBiotec 130-042-401). For B cells, purity was determined by flow cytometry using PE anti-B220 (eBioscience 12-0452-83). Purity was consistently found to be between 92% and 95% (data not shown). Complete media consisting of RPMI-1640 (Gibco 11875), 10mM HEPES (Gibco 15630), 1 mM Sodium Pyruvate (Gibco 11360), 1 mM non-essential amino acids (Gibco 11140), 0.055 mM β-mercaptoethanol (Gibco 21985), 100 units Penicillin/Streptomycin (Gibco 10378016) and .3 mg/ml glutamine was used to culture either B cells. B cells were stimulated with 5-10μg/ml of Fab Goat anti-mouse IgM, m-chain specific (Jackson Immunology Research#115-006-020) and / or 50ng/ml Recombinant Mouse BAFF / BLyS / TNFSF13B (R&D systems 2106BF).

Electrophoretic Mobility Shift Assay. Nuclear extracts were generated from B cells using high salt extraction. In brief, purified B cells were incubated with a low salt buffer (10mM HEPEs pH 7.9 (Gibco), 10mM KCl

(Thermo Fisher Scientific P217), 0.1mM EGTA (Sigma E-4378), 0.1mM EDTA (Thermo Fisher Scientific S312), 1mM DTT (Thermo Fisher Scientific BP172-5), 1mM PMSF (Sigma P7626), 5μg/ ml apoprotein (Sigma A1153), 5μg/ml leupeptin (Sigma L2884), 1μM pepstatin A (Sigma P5318) for 10 minutes on ice. Following this incubation, the cells were disrupted through the addition of NP-40 (US Biological N3500) to a final concentration of .5% and vortexing for 15 seconds. Nuclei were pelleted away from the cytoplasmic fraction by centrifugation at 15,000 rpm for 1 minute and the cytoplasmic fraction was pipetted into a separate tube. The remaining nuclei were disrupted by a 20 minute incubation at 4oC in a high salt buffer (20mM HEPES pH 7.9 (Gibco), 400mM NaCl (Thermo Fisher Scientific S671), 1mM EGTA (Thermo Fisher Scientific), 1mM EDTA (Thermo Fisher Scientific), 20% Glycerol (Thermo Fisher Scientific), 1mM DTT (Thermo Fisher Scientific), 1mM PMSF(Sigma)). The nuclear fraction was collected following centrifugation at 15,000 rpm for 5 minutes. Equal amounts of nuclear extracts (1ug) were pre-incubated for 20 minutes on ice in the presence or absence of antibodies specific for RelA (Santa Cruz Biotechnology sc-372), RelB (Santa Cruz Biotechnology sc-226), and cRel (Santa Cruz Biotechnologies sc-71) or in combination. Following the pre-incubation with antibodies, [γ–32P]ATP (GE health) radio-labeled probe derived from HIV-κB sequence: 5’-GCTACAAGGGACTTTCCGCTGGGGACTTTCCAGGGAGG-3’ was added and incubated at room temperature for an additional 15 minutes. The resulting

DNA/protein/antibody complexes were resolved by electrophoresis on a 5% non-denaturing polyacrylamide gel and exposed to storage phosphor screen (GE healthcare) overnight before image development on at Typhoon 9200 Variable Mode Imager (GE healthcare). Images were analyzed and quantitated using ImageQuant™ (GE Health).

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. The resulting lysates and extracts were run on either 10% SDS-PAGE gels or 5%-14% Criterion Tris-HCl Gel (Bio Rad). The following antibodies were used to identify the protein of interest: p65, cRel, RelB, Iκbα, Iκbε, actin, αtubulin (Santa Cruz Biotechnology), nfkb1 and nfkb2 (Dr. Nancy Rice). The resulting proteins were detected using the Bio-Rad ChemiDoc XRS System and SuperSignal West Femto Substrate Maximum Sensitivity Substrate (Thermo Scientific) to detect chemiluminescense released by HRP-labeled secondary antibodies.

IKK kinase assays. Assays were performed as described (Werner et al., 2005). Briefly, cytoplasmic extracts were collected and the IKK complex was immunoprecipitated with anti-IKKγ antibody, followed by addition of protein G sepharose beads. Beads were washed and subject to in vitro kinase assay containing ATPγ32, GST-IκBα(1-54). The reaction was stopped with SDS-sample buffer, and proteins were resolved by SDS-PAGE, and visualized by autoradiography or immunoblot.

Flow cytometry analysis of survival and B cell development. Purified B cells were stained with cultured in complete media with or without BAFF, at various time points B cells were collected and stained with 7AAD (Invitrogen A1310). The B cells were analyzed for survival using a C6 Accuri flow cytometer (BD Biosciences). Differences in cell viability was measured using FlowJo (Tree star inc.).

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) and analyzed using FlowJo software (Tree Star, Inc.) and FLOWmax (Shokhirev and Hoffmann, 2013)which determines maximum-likelihood cellular parameter sets for fraction of responding cells, times to division, and times to death for generations 0 to 10.

Computational modeling. This model is adapted from the previously published IκB – containing NFκB signaling models with the reactions that govern NFκB dimer generation. The input to this model is a numerical curve for NEMO-IKK2 activity and the outputs are free, nuclear RelA:p50, RelA:p52, RelB:p50, RelB:p52, cRel:p50, and cRel:p52 dimers. The association and dissociation rate constants between IκB s and NFκBs were adapted from a previous iteration of a B cell version of the IκB – NFκB signaling model.

This model represents the single-cell response of a population of B cells, and the variability of NFκB responsive cells is assumed to be in the activation of NEMO-IKK2. Based on population FACs experiments, a percentage of active responsive cells were assumed for each population in determining the output protein levels. Representative of the percentage of active B cells are as follows: anti-IgM activation of wild type B cells results in 20% active cells, BAFF activation of wild type B cells results in 50% active cells, co-stimulated B cells results in 30% active cells, while anti-IgM activation of *nfkb2*$^{+/-}$B cells results in 10% active cells. We defined a population of B cells to include 1000 simulations, with a percentage of on NFκB –responsive cells resulting from basal levels of NEMO-IKK2 and NIK, and the active cells have a log-normal population distribution of NEMO-IKK2 activity and increased NIK accumulation as defined below.

The ODEs were solved numerically using MATLAB version R2013a (The MathWorks, Inc.) with subroutine ode15s, a variable order, multi-step solver. Prior to stimulation, the system was allowed to equilibrate from starting conditions to a steady state, defined as showing no concentration changes greater than 1% over a period of 4000 minutes. Stimulus-induced perturbation from the steady state was accomplished by direct modulation of IKK activity via a numerical input curve representing IgM and BAFF stimulation (adapted from Werner 2008). Non-canonical activity was simulated by decreasing the

NIK degradation rate constant by 30-fold at 5 hours, resulting in increased NIK accumulation. MATLAB model codes are available upon request.

RNAseq. Total RNA was isolated from 50ng /ml BAFF stimulated wild type B cells isolated following 8 and 30 hours of culture. mRNA was extracted from 2 μg total RNA using oligo(dT) magnetic beads and fragmented at high temperature using divalent cations. cDNA libraries were generated using the Illumina TruSeq 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 by the BIOGEM core facility located at 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 CummRbund (31) was used to ascertain differential expression of genes. Gene differential FPKMs were obtained from the cuffdiff program in the Tuxedo RNA-seq analysis suite.

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

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CHAPTER 4:

Final remarks and future directions

Hematopoetic stem cells are capable of developing into the B cell. B cells first develop in the fetal liver, but in the adult they arise from the bone marrow, which was the focus on this thesis work. Outside the bone marrow, in the periphery, BAFF-R and BCR signals cooperate to support the development of a diverse B cell repertoire capable of mounting an immune response to a multitude of pathogenic species. Unlike cells of the innate immune system, which identify a limited quantity of pathogen associated molecular patterns (PAMPS), each B cell expresses a unique antigen receptor. Once a specific foreign antigen encounter occurs, massive and rapid clonal expansion soon follows, along with priming of antigen-experienced B cells and class-switch recombination (CSR). At the end of the response a subset of pathogen-specific cells must be safeguarded to provide memory. Therefore, the lifecycle of lymphocytes encompasses development, homeostasis for a finite time period, selection by antigens for expansion, and long-term survival of memory cells.

NFκB is involved in all stages of the B cell life-cycle. Peripheral B cell maturation, survival, and antigen-dependent differentiation are complex processes. This study tries to further understand the molecular cues that govern B cell fate at each of these distinct stages. Restrictions by NFκB on B cell development initiates in the bone marrow near the immature B cells stage. This is most likely due to the initial expression of BAFF-R on these cells which exerts its survival and developmental signals on immature and subsequent B

cell populations. Our thesis examines the functions of NFκB mediated through the BAFF receptor throughout two phases of B cell life cycle: 1) peripheral B cell development within the spleen and 2) activation following the encounter of antigen.

Targeted deletion of BAFF ligand or BAFF-R results in a partial block at the T1 → T2 transition, leading to severe deficiency of mature B cells (Harless et al., 2001; Schiemann et al., 2001). Conversely, if BAFF levels are experimentally elevated, the rigidity of transitional selection is “relaxed,” and cells that would normally be selected against, instead survive to join the FO or MZ pool (Stohl, 2005; Stohl et al., 2005). While BAFF is required for the development and survival of peripheral B cells, it is also implicated in over-aggressive immune response in autoimmune diseases like systemic lupus erythematosus (SLE). Soluble BAFF and APRIL are highly expressed in the serum of SLE patients, leading to autoreactive B cell proliferation and survival (Pers et al., 2005). B cells are therapeutic targets in SLE because they produce pathogenic autoantibodies and have multiple effector functions, including antigen presentation to T cells and cytokine production and migration to sites of inflammation(Lipsky, 2001). Thus, studying the underpinning mechanisms by which abhorrent BAFF signaling occurs, we cultivate our understanding of the pathology of autoimmunity, and possible identification of future pharmaceutical targets.

Within the context of homeostasis, we find that BAFF activates multiple NFκB dimers; RelA, RelB and cRel. Of these three, RelB and cRel in particular share interdependencies as single deletion has only a minor effects on B cell development, while compound deletion results in drastic reductions in mature populations, and a blockage at the T1 to T2 developmental stage. The activation of cRel by BAFF is an underappreciated finding, as the majority of previous studies focus primarily on the role of non-canonical mediators nfkb2 and RelB. Given the preeminent role of cRel in cell cycle entry and survival expression programs, two responsibilities paramount to proper BAFF-mediated B cell development; we question the activities of RelB under the control of BAFF signaling. Therefore, future work must include the delineation of distinct and overlapping gene programs for RelB and cRel in the BAFF signaling pathway. In addition, the current dogma of canonical NFκB activation may not apply for BAFF, as no NEMO activity is observed and little to no degradation of classical IκBα is seen. Further biochemical analysis may establish a single NIK-dependent, non-canonical mechanism which will permit the activation and release of all three NFκB family members RelA, RelB, and cRel.

Genetic rearrangements during maturation intrinsically predispose B cells to oncogenic mutations, however robust expansion program during B cell activation augments the risk of malignant disorders (Shaffer et al., 2002). Diffused large B cell lymphoma (ABC-DLBCL),which resemble mitogenically

activated peripheral B cells, contain high constitutive NFκB activity and NFκB target gene expression that includes BCL-2, IRF4, cFLIP and cyclin D2 (Davis et al., 2001). A high abundance of genetic mutations, which chronically activate the non-canonical NFκB pathway, was observed in multiple myeloma (Keats et al., 2007). In this plasma cell malignancy, BAFF, a potent activator of the non-canonical signaling was indeed shown to protect B cells from apoptosis by activating BCL-2 and BCL-XL expression (Moreaux et al., 2004).

Mice with inactivating mutations in cRel and RelA have normal numbers of mature B cells, but these cells are defective in their proliferative responses to BCR signaling and to LPS (Doi et al., 1997; Grumont et al., 1998;Kontgen et al., 1995). The proliferative defect in cRel–deficient B cells is reflected in a block at the G1 stage of the cell cycle (Grumont et al., 1998). However, optimal increases in cell numbers after BCR stimulation also involve inhibition of apoptosis, and NFκB activity has again been implicated. cRel–deficient B cells die by apoptosis in response to BCR ligation (Grumont et al., 1999; Grumont et al., 1998). Pro-survival genes such as BCL-2 family member A1 is a target of NFκB, and is induced after BCR stimulation. Ectopic expression of A1 in cRel–deficient B cells rescues the cells from apoptosis (Grumont et al., 1999). Thus, NFκB controls both proliferation and apoptosis in B cells to maximize the response to mitogens.

The oncogenic potential of cRel arose from the study of the avian Rev-T retrovirus, which contains v-rel as its sole gene which can transform and

immortalize a variety of chicken hematopoietic cell types from primary spleen and bone marrow cultures, including B- and T-lymphoid cells, myeloid cells, and dendritic cells *in vitro*(Gilmore, 1999; Gilmore et al., 2001; Rayet et al., 2003). Unlike other human NFκB family members (p50, p52, RELA, RELB), overexpression of chicken, mouse, and human cRel can also transform chicken lymphoid cells in vitro(Rayet et al., 2003).Thus, making the necessity for strict regulation of cRel vital. In fact multiple, redundant mechanisms of control exist. The accepted mechanism of regulation is through interaction with classical Iκbs, α and ε to sequester the cRel complexes in the cytoplasm and blocks its ability to bind DNA. However, regulation of NFκB has been shown to be cell-type and stimulus specific (Basak et al., 2007; Hoffmann et al., 2002; O'Dea and Hoffmann, 2010; Shih et al., 2012;Shih et al., 2009).

In chapter three, we find that the non-classical IκB, IκBδ is a regulator of cRel in B cells following anti-IgM stimulation. Unlike the homestatic model in which BAFF-R's transcriptional effector is RelB:p52, whose generation is supported by tonic BCR signals that induce nfkb2 expression to replenish depleted p100/p52 pools (Stadanlick and Cancro, 2008; Stadanlick et al., 2008), we show that during B cell expansion, BAFF-R functions to counteract high p100 levels which form the IκBδcomplex with cRel to switch off the mitogenic program. Thus IκBδ provides a "brake" in the B cell expansion programs. Consistent with the B cell proliferation and survival defects, cRel null mice have small / irregular germinal centers (Cariappa et al., 2000). NFκB

signaling is critical for isotype switching, consequently making persistent canonical NFκB activity indispensable in mounting proper immune responses (Hayden et al., 2006). BAFF has been implicated in plasma cell survival (O'Connor et al., 2004), and it enhances both T cell-dependent and independent humoral immune responses (Do et al., 2000; Litinskiy et al., 2002). Our work shows that BAFF-R signals, independent of its survival functions during development, will remove the "brake" on cRel via the NIK-dependent non-canonical pathway, allowing for persistent cRel antigen driven activity. The result is a "super-activation" of cRel allowing for prolonged B cell expansion. Further studies may examine the presence of cRel and IκBδ in GC B cells, plasma and memory cells. In our study, the nfkb2 heterozygous mice was used to examine effects of Iκbδ on cRel in BCR stimulated conditions. These mice, due to decreased p100 expression at the protein and gene level, are shown to display an enhanced capacity for B cell proliferation following anti-IgM stimulation. Future analysis of these mice should include the ability to mount antibody responses, GC initiation, and plasma cell abundances.

While this thesis has focused on mechanism of BAFF activation through NFκB, additional signaling pathways contribute to the BAFF function. Most prominently would be the phosphatidylinositol (PtdIns) 3-kinase (PI3K) pathway (Baracho et al., 2011; Rickert et al., 2011). BAFF treatment leads to the rapid phosphorylation of the serine/threonine kinase Akt as well as the induction of genes involved in the regulation of glycolysis and cell cycle

progression (Patke et al., 2006). This activation of Akt is dependent upon PI3K, as treatment of B cells with the PI3K inhibitor LY294002 blocked the BAFF-induced activation of Akt (Patke et al., 2006; Woodland et al., 2008). Mcl-1, a short lived member of the Bcl-2 family required for the development of hematopoietic stem cells and for the maintenance of peripheral B and T cells (Opferman et al., 2005; Opferman et al., 2003).has been shown to be involved in BAFF signaling (Giltiay et al., 2010; Woodland et al., 2008). Further, the PI3K–AKT pathway (downstream of BAFF) positively regulates transcription of the Mcl-1 gene (Wang et al., 1999).

The initial construction of the mathematical model for the NFκB signaling module was motivated by the intricate dynamic control apparent in time course experiments (Hoffmann et al., 2002). Since then, the mathematical model has proved to be a useful tool for dissecting the mechanisms that control NFκB dynamics and for guiding experimental design (Basak et al., 2007; O'Dea and Hoffmann, 2010; Shih et al., 2012; Shih et al., 2009; Werner et al., 2005). This was also the case for our study in B cells, as we have established a computational model which recapitulates the regulation an dynamics of NFκB within the framework of antigen stimulation. However, the NFκB signaling system is embedded within a larger network, which includes PI3K and mitogen activated protein kinase (MAPK) family (Dal Porto et al., 2004; Kurosaki et al., 2010), to determine proliferative and developmental responses to BAFF and BCR signals. Future steps should

include integration of these signaling modules. The utility of an integrated NFκB-PI3K-MAPK model is to enable a quantitative understanding of how B cells integrate multiple diverse signals to produce specific functional responses (Basak et al., 2012). Computational analysis combined with biochemical and genetic experiments may establish key mechanisms for crosstalk in specific biological settings, such as B cell differentiation and immune responses (Basak et al., 2012). Thus, ultimately impact our understanding of lymphocyte dynamics in infection and vaccine development.

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