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Control of B Lymphocyte Apoptosis by the Transcription Factor NF-κB

Review

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Summary

B cells maintain homeostasis by balancing cell viability and cell death. B lymphocytes are susceptible to mitochondria- and receptor-initiated cell death at various stages of peripheral differentiation and during immune responses. The inducible transcription factor NF-κB enhances cell viability by activating genes that counteract both cell-death pathways. This review uses characteristic features of NF-κB activation and downregulation to provide insight into the regulation of B cell apoptosis in the periphery. In particular, the temporal patterns of NF-κB induction, differences between Rel family members, and the intersection between canonical and noncanonical signaling pathways in keeping B cells alive are discussed.

Regulated cell proliferation and cell death are central to lymphocyte development and the immune response. The diversity essential for pathogen recognition is generated by the assembly of antigen-receptor genes from a random assortment of gene segments via V(D)J recombination. The price of ensuring diversity is that primary recombination events produce a range of receptor specificities of which only few are useful to the individual. Cells that express useless, or harmful, receptors are weeded out via altered receptor specificity or programmed cell death. The ability to expand the right clone for effective immune responses comes with the necessity of removing unwanted cells once the danger has passed. Furthermore, the process of cell elimination must be tempered so that some cells that retain memory of the encounter are preserved, yet it must be efficient enough so as not to leave behind cells that may cause autoreactivity.

Because of their importance in maintaining lymphocyte homeostasis, mechanisms of cell death, and how they are countered by survival signals, have been extensively studied. These studies have identified genes that participate in, or inhibit, two major death-inducing pathways in lymphocytes. One pathway, referred to as cell intrinsic, disrupts mitochondrial integrity (Green and Kroemer, 2004; Strasser, 2005) and leads to cytochrome c release and caspase activation. The other pathway, referred to as cell extrinsic, is initiated at cell-surface death receptors such as Fas and can circumvent mitochondria to directly activate effector caspases (Bidere et al., 2006; Lavrik et al., 2005). What is less clear is how the balance between apoptosis-inducing signals and antiapoptotic gene expression is achieved to produce optimal immune responses. For example, it is readily apparent that if cell death occurs too soon, the immune response will be ineffective; conversely, a prolonged immune response is likely to cause inflammatory problems. Thus, understanding how the life or death choice is regulated in lymphocytes is essential for future manipulation of dysregulated immune responses. The objective of this review is to address the problem of balance in B lymphocyte death from the perspective provided by the properties of the transcription factor NF-κB.

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NF-κB Regulation

The inducible transcription factor NF-κB (Hayden and Ghosh, 2004) protects against both forms of death by activating the transcription of genes that encode antiapoptotic proteins. From the demonstration more than a decade ago of increased sensitivity of NF-κB-deficient cells to tumor necrosis factor α (TNFα) or gentoxic-stress-induced death to more recent studies that implicate NF-κB activation in tumorigenesis (Karin, 2006; Pikarsky and Ben-Neriah, 2006), the antiapoptotic function of NF-κB has been documented in numerous cell types and in response to diverse stimuli. NF-κB activation involves phosphorylation of inhibitory IκB proteins by the IκB kinases α and β [IKKα and IKKβ (Bonizzi and Karin, 2004)], leading to IκB degradation and release of DNA-binding NF-κB, which translocates to the nucleus to activate gene transcription. The canonical activation pathway, utilized by inducing agents such as TNFα, IL-1, lipopolysaccaride (LPS), and antigen receptors, activates IKKβ with resultant degradation of IκBα, IκBβ, and IκBε. This pathway primarily activates NF-κB heterodimers consisting of p50 (NFκB1) associated with REL-A(p65) or REL. The noncanonical pathway, utilized by lymphotoxin β, B cell-activating factor of the TNF family (BAFF), and CD40, activates IKKα. This leads to phosphorylation and proteolytic processing of the C-terminal ankyrin domain of p100 and results in the release of p52 (NFκB2)-REL-B heterodimers to activate gene expression. Many NF-κB-inducing stimuli simultaneously trigger cell death, whereas genes that are activated by NF-κB are recurrently implicated in protecting against cell death. Thus, the regulation of NF-κB responses is required for striking the right balance between cell viability and cell death. Because rules that govern the choice will very likely vary with cell-types, inducing regimens, cell-cycle status, etc., it is imperative to understand NF-κB-dependent anti-apoptosis within specific biological contexts.

Temporal Control of NF-κB Responses

The transient nature of many forms of NF-κB activation is likely to be an important determinant of the duration of cell viability. This feature, sometimes referred to as postinduction repression, has been known for many years in the context of NF-κB induction by TNFα. Several mechanisms contribute to ensuring transient NF-κB induction. One of the most important is via upregulation of IκBα gene transcription by REL-A(p65), which leads

to de novo IκBα protein synthesis. The newly synthesized IκBα protein is proposed to enter the nucleus, associate with Rel proteins, and export them to the cytosol to terminate NF-κB-dependent gene transcription and re-establish the resting state of cells (Banerjee and Sen, 2006). Consequently, nuclear NF-κB lifetime is prolonged in IκBα-deficient cells, or in the absence of protein synthesis. Even this simple downregulatory pathway is further modulated by posttranslational modification of Rel proteins. Acetylation of REL-A(p65) inhibits its interaction with IκBα and prevents its removal from the nucleus, thereby resulting in increased NF-κB-dependent transcription. NF-κB-dependent inducible gene transcription is also regulated by nuclear proteasomal degradation of REL-A(p65) (Saccani et al., 2004). This regulatory pathway has been most clearly demonstrated on cytokine gene promoters, where recruitment of proteasomal components to the promoter suggests that the promoter-bound transcription factor is degraded to terminate a response. Interestingly, REL-A(p65) is targeted for nuclear degradation via phosphorylation by IKKα (Lawrence et al., 2005).

In addition to active depletion of the transcription factor from the nucleus, mechanisms are in place to ensure that the re-generated cytosolic pool is not efficiently re-activated. One NF-κB target gene induced by TNFα is A20. This is a ubiquitin ligase that ubiquitinates and marks for proteasomal degradation receptor-interacting protein (RIP) (Boone et al., 2004; Wertz et al., 2004), which is essential for TNFα receptor signaling to the IKK complex. In the absence of RIP, TNFα signaling to NF-κB is temporarily attenuated. The importance of this downregulatory mechanism is evident from the systemic inflammation seen in A20-deficient mice (Lee et al., 2000). An analogous situation occurs in T lymphocytes stimulated via the T cell receptor (TCR). TCR signals activate NF-κB via a heterotrimeric complex consisting of the CARD-domain proteins Bcl-10 and CARMA1, together with MALT1 (Weil and Israel, 2006). In this pathway, Bcl-10 is targeted for lysosomal degradation by a presently unknown mechanism (Scharschmidt et al., 2004). The consequent reduction in TCR signaling to IKKβ probably contributes to transient REL-A(p65) induction in $CD4^+$ T cells (Mittal et al., 2006). It is important to note that these downregulatory mechanisms selectively affect the inducing stimulus. That is, a T cell that has attenuated TCR signaling to NF-κB by degrading Bcl-10 is still responsive to NF-κB induction by a different pathway, such as TNFα or IL-1.

Finally, NF-κB-dependent gene expression may also be terminated via the generation of NFκB1(p50) homodimers, which lack classical transcription-activation domains. These proteins may serve as simple repressors by obstructing DNA binding of transcriptionally active Rel complexes that contain REL-A(p65) or REL. Alternatively, p50 homodimers may recruit histone deacetylases (HDACs) to NF-κB-dependent promoters to turn off genes by epigenetic means (Williams et al., 2006; Zhong et al., 2002). Like NF-κB-dependent induction of A20 or IκBα genes, this also represents a feedback-inhibitory mechanism because the gene encoding p105, the precursor to p50, is an NF-κB target. Thus, multiple pathways ensure that the NF-κB response is short-lived, although the relative importance of each pathway will probably vary with cell type and the initiating stimulus.

However, not all inducers lead to transient NF-κB activation. Among the best characterized is the long-term response to lipopolysaccharide (LPS) via toll-like receptor (TLR) 4. This has recently been shown to be due to two independent pathways of NF-κB activation downstream of TLR4 (Covert et al., 2005; Werner et al., 2005). Although the NF-κB response to each pathway oscillates as a result of IκBα feedback (Hoffmann and Baltimore, 2006; Hoffmann et al., 2002), the temporal distinction between them ensures continued NF-κB activity. The duration of NF-κB has been modeled as a function of the intensity and duration of IKKβ activity (Werner et al., 2005). Transient NF-κB inducers, such as TNFα, lead to kinetically rapid activation and de-activation of IKKβ. Long-term inducers, such as LPS, lead to kinetically slower and lower levels of IKKβ activation; this mechanism may also apply to the persistent NF-κB activation noted in certain classes of B lymphomas (Davis et al., 2001). Importantly, variation between these extremes provides the means for generating unique kinetic patterns of NF-κB responses.

NF-κB Kinetics Affect Cellular Apoptosis

Two recent studies exemplify the intersection between NF-κB cell biology and the regulation of apoptosis in immune cells. Lawrence et al. (2005) showed that macrophages from IKKα-deficient mice produced higher levels of inflammatory cytokines in response to LPS treatment. They traced the abnormality to extended duration of nuclear REL-A(p65) in mutant as compared to normal macrophages. In this case the kinetics of REL-A(p65) downregulation from the nucleus (during continued LPS stimulation) was altered because nuclear p65 was not targeted for degradation in IKKα-deficient macrophages. In addition to cytokine gene expression, NF-κB-dependent antiapoptotic gene expression was also elevated in these cells, resulting in extended cell viability. The combination of elevated cytokine production for longer periods of time translated into increased morbidity of IKKα-deficient mice in response to group B streptococcus or endotoxic shock.

In $CD4^+$ T cells, continuous TCR signals first induce and then downregulate REL-A(p65) from the nucleus (Mittal et al., 2006). Loss of nuclear REL-A(p65) coincides with the onset of Fas-initiated cell death, suggesting that NF-κB-dependent antiapoptotic gene expression regulates cell viability. Consistent with this idea, expression of three out of several putative NF-κB-dependent antiapoptotic genes follows the kinetics of NF-κB induction and downregulation. Although *Bcl2l1* (Bcl-X) is one of these genes, ectopic expression of $Bcl\text{-}X_L$ does not alter the kinetics of cell death, probably because Fas-initiated cell death bypasses the mitochondria. Expression of one of the other genes, *Gadd45b*, however, prolongs the viable phase of the cells. Thus, transient NF-κB activation results in transient expression of antiapoptotic genes in these cells, and programmed cell death occurs when these genes are downregulated. Both observations suggest that the duration of NF-κB activation has important cellular consequences.

Peripheral B Cell Survival

B cells that emerge in the periphery after completing antigen receptor gene rearrangements in the bone marrow undergo further differentiation to become mature, functional B cells. The most immature B cells in the periphery, also referred to as transitional B cells, express the AA4 cell-surface marker and are further subdivided into T1-T3 subsets on the basis of IgM, CD21, and CD23 expression (Thomas et al., 2006). A hallmark of immature B cells, in particular T1 cells, is their extreme sensitivity to apoptosis (Chung et al., 2003). Indeed, the prevailing view is that T1 cells are programmed to die unless positively selected to survive and differentiate further (Cancro and Kearney, 2004; Levine et al., 2000). This subset is also the target of negative selection signals which eliminate cells that express self-reactive BCRs. Cell death by neglect or negative selection occurs via the mitochondrial pathway by the action of proapoptotic Bcl-2 family members Bak and Bax and the BH3 domain-only protein Bim (Enders et al., 2003; Oliver et al., 2006; Takeuchi et al., 2005). In mice with B cell-specific double deletion of Bak and Bax, the numbers of immature $AA4^+$ and mature follicular B cells are higher than in controls, and these cells resist multiple death-inducing stimuli, including BCR cross-linking and cytokine withdrawal, in vitro. The phenotype of Bim-deficient mice is similar, although the effects are less accentuated than in the Bak and Bax double deficiency, indicating partial compensation for the absence of Bim.

NF-κB Regulation of Immature B Cell Survival

A role for NF-κB in immature B cell survival is evident from mouse mutations in Rel proteins (Gerondakis and Strasser, 2003) and signaling intermediates in the NF-κB activation pathway (Siebenlist et al., 2005). *Rel* and *Rela* double deficiency blocks peripheral B cell differentiation at the immature T1 stage, and cells that remain are highly susceptible to death in vitro (Grossmann et al., 2000). The developmental defect is due to reduced Bcl-2 expression and is overcome by transgenic expression of this gene. Single deficiency of either REL or REL-A(p65) does not have this phenotype, indicating that each of these Rel family members functionally compensates for the absence of the other. Double deficiency of *Nfkb1* and *Nfkb2* genes blocks differentiation at the immature T2 stage (Claudio et al., 2002). As with other single-gene deficiencies of Rel family proteins, the steady-state phenotypes of either *Nfkb1* or *Nfkb2* gene deletions is subtle with regard to follicular B cells; however, the number of marginal-zone B cells is reduced (Cariappa et al., 2000) in the absence of *Nfkb1*, and remaining cells in either single-gene deficiency are sensitive to apoptosis (Franzoso et al., 1998; Grumont et al., 1998). Because p50 (derived from *Nfkb1*) and p52 (derived from *Nfkb2*) proteins are downstream effectors of the canonical and noncanonical NF-κB signaling pathways, respectively, these observations suggest that both pathways contribute to survival and differentiation of immature B cells. Accordingly, mutation of signaling kinases in each NF-κB pathway also blocks the generation and maintenance of mature B lymphocytes. IKKα-deficient precursors, whose only discernible NF-κB defect is a reduced amount of p52, reconstitute fewer B cells than normal precursors because of a block at the T2 stage of differentiation (Kaisho et al., 2001; Senftleben et al., 2001). Downregulation of the canonical pathway by conditional deletion of *Ikbkb* (Li et al., 2003; Pasparakis et al., 2002) or *Ikbkg* (Sasaki et al., 2006) results in a developmental block at the T1-to-T2 transition. The stronger phenotype of single IKK deficiencies compared to NFκB1 or NFκB2 deficiency probably reflects similar DNA-binding properties of p50 and p52 homodimers, respectively, rather than functional overlap between IKKα and IKKβ.

B Cell Receptor Regulation of B Cell Survival

The B cell receptor (BCR) provides survival signals essential for maintaining the mature B cell pool. This was first demonstrated by inducible deletion of the *Igh* gene in the periphery; this deletion leads to a loss of B cells (Lam et al., 1997). These observations were further substantiated recently by conditional deletion of either *Igh* or the signal-transducing *Cd79a* (Igα) genes (Kraus et al., 2004; Meffre and Nussenzweig, 2002). A role for BCR signaling is evident in the most immature T1 transitional B cells. Tze et al. (2005) found that deleting *Cd79a* in immature IgM^{hi} B cells resulted in de-differentiation as evidenced by transcriptional activation of genes normally expressed in pro-B cells. They proposed that the BCR maintained the differentiation state of immature B cells. In addition, deficiency of several cytoplasmic molecules implicated in BCR signaling, such as Btk, PLCγ, PI3K (Su et al., 2004), and the adapter proteins BLNK and BCAP (Koretzky et al., 2006; Kurosaki, 2002; Yamazaki and Kurosaki, 2003) attenuates peripheral differentiation at the immature stage, although the impact of each gene mutation varies.

The close correspondence between a requirement for canonical NF-κB components and BCR signaling in transitional cells suggests that the BCR may induce NF-κB in these cells. However, although the case for BCR signaling in transitional cells is strong (Fuentes-Panana et al., 2004), the evidence that these signals culminate in NF-κB activation is weaker. The idea that BCR signals induce NF-κB is supported by, in addition to the phenotype of NF-κB-deficient mice, the observation that the maturation defect in BCAP-deficient B cells involves NF-κB and, in particular, REL (Yamazaki and Kurosaki, 2003). In contrast, genetic deletion of CARMA1, Bcl-10, or MALT1 does not substantially affect follicular B cell maturation (Hara et al., 2003; Newton and Dixit, 2003; Ruefli-Brasse et al., 2003; Ruland et al., 2001; Xue et al., 2003), which has been taken to indicate that BCR-induced survival signals are not mediated by NF-κB. Although these proteins are essential components of antigen-receptor signaling to NF-κB in mature B cells, we cannot rule out the possibility that BCR-induced survival signals activate NF-κB by a route that does not involve the Bcl-10-CARMA-1-MALT-1 complex. Mechanistically, this may be because the BCR in immature cells is not associated with lipid rafts (Chung et al., 2001), or it may simply be because the nature of tonic signaling is different from what is recapitulated by BCR crosslinking in vitro (Monroe, 2006). Functionally, the result may be reduced IKKβ activation that allows persistent NF-κB-dependent antiapoptotic gene expression that is essential for maintaining B cell viability. Overall, it remains plausible that BCR signals result

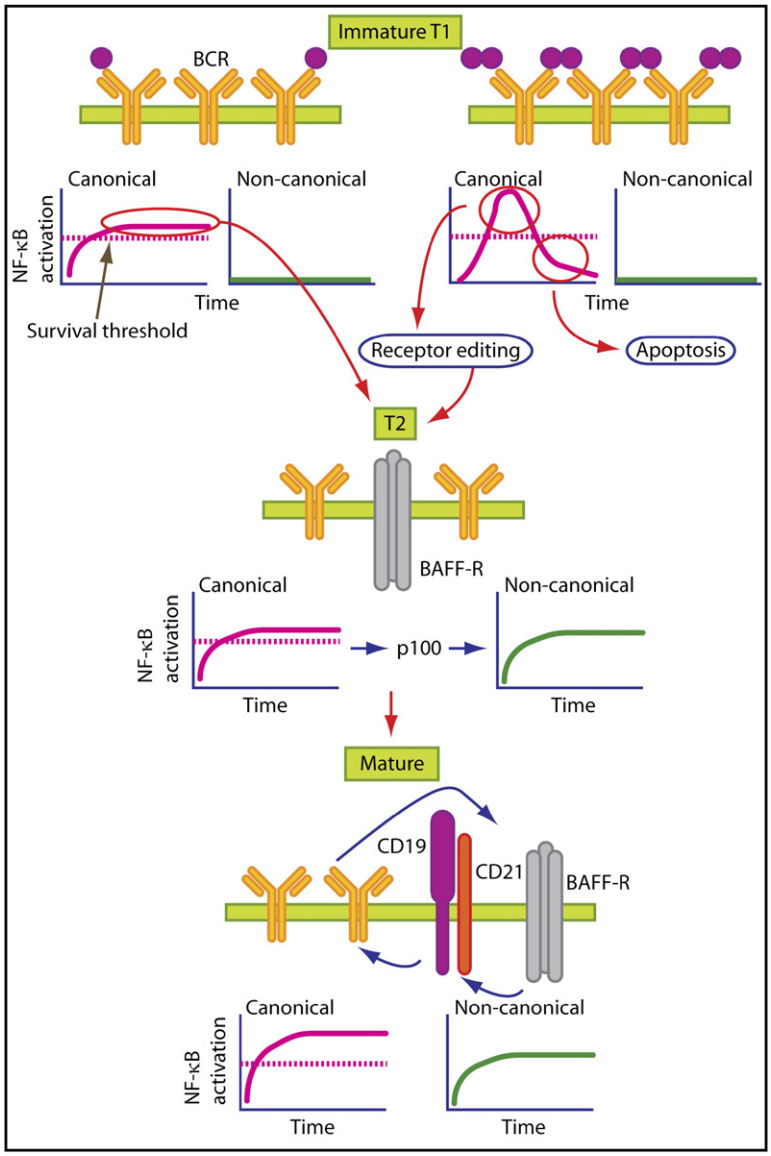

Figure 1. Proposed NF-κB Control of Peripheral B Cell Maturation and Maintenance

BCR signals in T1 transitional cells (top left) may activate canonical NF-κB proteins, either in response to substrate recognition or by "tonic" signaling, for extended duration as represented in the left graph (Y axis = NF-κB, X axis = time, dotted line represents the survival threshold). At this stage there is minimal noncanonical signaling, as represented by the right graph. Extensive BCR crosslinking (top right) leads to acute, but transient, NF-κB induction; during the downregulation phase, NF-κB amounts may drop below the survival threshold, resulting in apoptosis. While NF-κB expression is high, cells survive and have the opportunity to undergo receptor editing, particularly in the protective microenvironment of the bone marrow. NF-κB-mediated survival allows progression to the T2 stage (middle), where BAFF-R is first expressed. Continued canonical signaling via the BCR generates p100 (NFκB2), which serves as the substrate for the IKKα-dependent noncanonical NF-κB pathway initiated at BAFF-R. This induces p52-REL-B complexes as represented by the right graph (green line). BAFF-R signals activate multiple survival pathways, such as Akt, Pim2, and PKCδ, and downregulate the proapoptotic protein Bim. In addition, BAFF-R signals induce CD21 gene expression to generate the BCR coreceptor complex (bottom). The presence of this complex may enhance BCR signals to elevate persistent canonical NF-κB (left graph) and NF-κB-dependent survival-gene expression. Increased BCR signals may feed into the BAFF-R pathway by increasing BAFF-R expression (indicated by arrow between BCR and BAFF-R). As a consequence, a self-reinforcing loop is established, with the BCR enhancing the BAFF-R pathway and vice-versa. Perhaps the loop is held in check by BAFF concentrations and BCR substrate recognition being limited in vivo.

in immature B cell survival by inducing NF-κB by the canonical pathway. In this scenario, T1 cells that migrate to the periphery lose bone-marrow-derived survival signals, thus tilting the balance in favor of mitochondria-initiated apoptosis. A proportion of these cells are rescued to differentiate further if they receive a BCR signal of sufficient strength to counteract cell-intrinsic apoptosis by inducing NF-κB-dependent Bcl-2 family genes (Figure 1, top left).

This kind of survival signaling is overridden in the face of extensive BCR crosslinking, which leads to apoptosis of immature T1 cells. Defective NF-κB activation in these cells has been suggested as a possible cause for their sensitivity to cell death based on reduced induction of Bcl-X and A1 genes (Feng et al., 2004; Su and Rawlings, 2002). Moreover, reduced PKC activation, as a consequence of reduced PIP_2 hydrolysis (King et al., 1999), may also result in ineffective NF-κB activation in these cells. In this model, BCR crosslinking in T1 cells activates proapoptotic pathways, but not antiapoptotic mechanisms to neutralize them. However, IκBα degradation in response to BCR crosslinking is comparable in T1, T2, and mature B cells (W. Khan, personal communication). This suggests that BCR crosslinking may induce acute, but transient, NF-κB activation (Figure 1, top right). While NF-κB is up, the cells survive; however, during the downregulation phase initiated by REL-A(p65)-dependent IκBα gene transcription, the bolus of newly synthesized IκBα may reduce NF-κB amounts below the threshold required to maintain effective antiapoptotic gene expression. When this happens, the proapoptotic activity of proteins such as Bim dominate, and this leads to cell death. Self-recognition by immature B cells in the bone marrow also initiates receptor editing (Louzoun et al., 2002), particularly at the *Igk* light-chain gene locus. The heightened NF-κB response may also induce RAG gene expression essential for this process (Amin and Schlissel, 2005; Verkoczy et al., 2005), and the κ locus itself may be targeted via NF-κB binding to the κ gene intron enhancer. The transient nature of NF-κB activation in response to BCR crosslinking would allow a finite window of opportunity for these processes

to occur before cell death. Previously documented protective effects of the bone-marrow microenvironment (Sandel and Monroe, 1999) may prolong NF-κB activation or provide compensatory means to maintain cell viability after NF-κB downregulation.

BAFF-R Regulation of B Cell Survival

In addition to the BCR, mature B cell survival requires the ligand-receptor pair BAFF and BAFF-receptor (BAFF-R) (Crowley et al., 2005; Woodland et al., 2006). An essential role of BAFF in vivo was inferred from the markedly reduced numbers of B cells in mice that lack either the ligand or the receptor. Additionally, treatment of normal mice with receptor fusion proteins that sequestered soluble BAFF also led to rapid loss of B cells. Based on in vitro studies, these defects were attributed to BAFF-dependent B cell survival (Smith and Cancro, 2003). The survival defect in the absence of BAFF is largely overcome by transgenic expression of Bcl-X_L, indicating that BAFF-R signals attenuate mitochondria-initiated death (Amanna et al., 2003). During peripheral differentiation, BAFF-R is highest at and beyond the T2 transitional stage; accordingly, differentiation is compromised between the T1 and T2 stages in BAFF-, or BAFF-R-, deficient mice. The observation that BCR crosslinking on mature B cells upregulates BAFF-R expression (Smith and Cancro, 2003) raises the possibility that BCR activation in T1 cells in vivo may induce BAFF-R expression in addition to NF-κB-dependent survival genes, thereby allowing progression to the T2 stage (Figure 1, middle). The strength of the BCR signal would likely be key in determining whether BAFF-R expression, and BAFF-R-dependent survival signals, could overcome T1 cell death initiated at the BCR.

Multiple pathways regulate survival by BAFF beyond T2 transitional cells. One of the first to be characterized was the noncanonical NF-κB pathway that generates p52-REL-B nuclear complexes by IKKα-dependent NF-κB processing (Claudio et al., 2002; Kayagaki et al., 2002). However, antiapoptotic gene targets of p52-REL-B heterodimers remained unclear until recently. The studies of Enzler et al. (2006) now show that expression of Pim-2, an antiapoptotic serine-threonine kinase, is strictly dependent on IKKα and p52, implicating this gene as a possible downstream target of p52-REL-B. The block in peripheral B cell differentiation, and the absence of BAFF-induced cell viability in vitro, in cells that lack IKKα further underscores the importance of this pathway (Enzler et al., 2006). BAFF-induced expression of NF-κB target genes *Bcl2l1* (Bcl-X) and *Bcl2a1a* (A1) is also reduced in IKKα- and p52-deficient B cells, but the functional significance of this decrease is unclear. Instead, Woodland and colleagues find that Mcl-1 is essential for BAFF-R-dependent survival signaling in vitro (R.T. Woodland, personal communication).

In addition to activating noncanonical NF-κB, BAFF signals attenuate cell death by directly targeting Bim. BAFF-initiated ERK activation results in Bim phosphorylation and degradation (Craxton et al., 2005), whereas BAFF-induced Akt activation (Patke et al., 2006) may reduce *Bcl2l11* (Bim) gene transcription by blocking the transcription factor. Both mechanisms lower Bim levels and thus favor cell survival. Additionally, Akt is known to increase NF-κB induction via the canonical pathway in various cell types. Therefore, it is possible that BCR-induced NF-κB induction, and NF-κB-dependent survival gene expression, is enhanced by Akt activated via BAFF-R. BAFF also activates Pim-2 gene expression as described above, and this is linked to cell survival via inactivation of the proapoptotic protein Bad (Fox et al., 2003). Interestingly, a closely related protein, Pim-1, has been previously implicated in NF-κB function (Hammerman et al., 2004; Zhu et al., 2002). Finally, cytoplasmic redirection of PKCδ is another mechanism by which BAFF regulates B cell survival (Mecklenbrauker et al., 2004). This pathway is also activated by constitutive IKKβ expression in BAFF-R-deficient mice, suggesting a link to NF-κB (Sasaki et al., 2006). Overall, BAFF-BAFF-R signals attenuate several mitochondria-targeted proapoptotic mechanisms and may thereby create a milieu in which antiapoptotic genes function more effectively. The close coincidence of multiple BAFF-induced survival pathways with previously identified NF-κB targets indicates that BAFF-R-initiated signals intersect directly and indirectly with NF-κB to regulate peripheral B cell survival.

Mechanisms of BCR- or BAFF-R-initiated survival signals must explain the central fact that both pathways are simultaneously required for B cell survival in vivo. Several NF-κB-dependent synergistic interactions between the two pathways can be envisaged based on available data. The BCR may feed into the BAFF-R pathway by regulation of BAFF-R expression and by canonical NF-κB-dependent p100 gene transcription to create a pool of p100-REL-B complexes that are targeted by BAFF-R-dependent activation of the noncanonical pathway. As suggested above, this part of the synergy may be initiated in T1 cells. BAFF-R signals at the T2 stage have the potential to reinforce the canonical NF-κB pathway by activating Akt and CD21 gene expression (Gorelik et al., 2004). Akt is known to accentuate NF-κB induction, and CD21 may participate by lowering the threshold of BCR signaling via the CD19/CD21 coreceptor complex (Rickert, 2005). The net effect of such mutual reinforcement is elevated NF-κB, and NF-κB-dependent antiapoptotic gene expression, in mature cells (Figure 1, bottom). It is tempting to speculate that the resulting high basal expression of NF-κB is what is visualized as constitutive NF-κB uniquely in B lymphocytes. In this scenario canonical NF-κB is the major mediator of antiapoptotic gene expression in a cellular environment where proapoptotic pathways have been downregulated by BAFF-R signals; thus, loss of either signaling pathway leads to cell death.

Recently, Rajewsky and colleagues provided strong evidence that activation of the canonical NF-κB pathway restores peripheral B cell differentiation in BAFF-R-deficient mice (Sasaki et al., 2006). They expressed a constitutively active form of IKKβ in the B lineage, and this resulted in increased nuclear expression of canonical NF-κB components REL-A(p65) and REL, but not p52. This genetic manipulation restored peripheral B cell differentiation in BAFF-R-deficient mice, suggesting that the canonical NF-κB pathway was sufficient for B cell survival. These observations are consistent with the crux of the above model, in which the ultimate result of BAFF-R and BCR synergy is canonical NF-κB activation. Mechanistically, constitutive IKK-2 expression may

circumvent the need for BAFF-R-dependent synergy in activating the canonical pathway via the BCR.

B Cell Survival during the Immune Response

The mitochondrial cell-death pathway continues to play a central role in B cell survival at multiple stages of the immune response. B cells respond to antigen in the presence, or absence, of T cell help. Type-II T-independent antigens initiate responses by extensive crosslinking of the BCR, leading to short-term antibody production without significant affinity maturation. The initial phase of the T-independent responses has been mimicked in vitro by BCR crosslinking with anti-immunoglobulin (anti-Ig). An extensive literature documents the effects of anti-Ig, particularly the elucidation of cytosolic signaling pathways mediated by kinases and phosphatases (Dal Porto et al., 2004; Jumaa et al., 2005). Farther downstream, cell proliferation is one parameter used for reading out B cell activation. Although anti-Ig stimulates B cell proliferation in vitro, a substantial proportion of mature B cells also die in response to BCR crosslinking. The effect, however, is less pronounced than in immature B cells. This form of death is reduced in transgenic mice that express antiapoptotic Bcl-2 family members, indicating that it is driven by mitochondria (Enders et al., 2003). Pro-apototic Bim is again a key player in this process; it is increased by transcriptional as well as posttranscriptional mechanisms in response to BCR crosslinking (Enders et al., 2003). Thus, Bim-deficient B cells are largely protected against anti-Ig-induced death regardless of their peripheral differentiation stage.

The NF-κB family member REL plays a crucial role in alleviating BCR-induced death of mature B cells. Phenotypically mature B cells from *Rel*-deficient mice die in response to BCR crosslinking in a manner reminiscent of immature B cells. This is because of defective Bcl-x and A1 gene transcription, and cell death can be averted by transgenic Bcl-2 or Bcl-X_L expression (Grumont et al., 1999; Owyang et al., 2001). Although it is likely that Bim is the major death-inducing protein in REL-deficient B cells, this has not been directly proven. These observations indicate that REL-dependent Bcl-X_L or A1 induction counteracts Bim-induced mitochondrial dysfunction in wild-type B cells. If the outcome favors REL-dependent gene expression, the cells survive and proliferate; if not, Bim-induced death ensues. The extent of B cell death in response to BCR suggests that the outcome disproportionately favors Bim-induced death, unless additional antiapoptotic signals neutralize it. Signals that enhance B cell viability during anti-Ig crosslinking include LPS, BAFF-R, and CD40 (Smith and Cancro, 2003); each of these stimuli can alter NF-κB-dependent antiapoptotic gene expression (Figure 2), or directly downregulate Bim, for example via Erk-dependent phosphorylation and degradation. In addition to cell survival, REL is also essential for BCR- or CD40- (see below) induced B cell proliferation in vitro. This has been attributed to REL-dependent induction of E2F3 and cyclin E to mediate G1-S progression (Cheng et al., 2003).

What distinguishes REL from other NF-κB family members, particularly REL-A(p65), in its ability to provide survival signals to BCR-activated mature B cells? One possibility is that Bcl-X_L and A1 gene promoters are uniquely responsive to REL in B cells. This appears

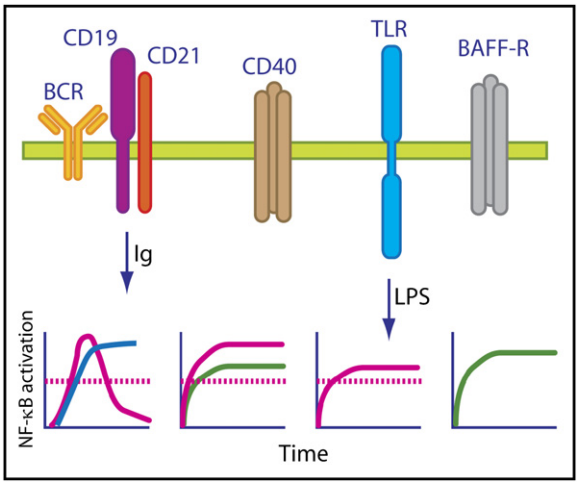

Figure 2. Patterns of NF-κB Activation by B Cell Surface Receptors

The graphs summarize patterns of NF-κB activation by four important NF-κB-inducing cell-surface receptors (Y axis = NF-κB level; X axis = time; red line = NF-κB induced by the canonical pathway; green line = NF-κB induced by the noncanonical pathway; blue line = REL induced by de novo gene expression). More than one receptor may be activated during a typical immune response (for example, BCR plus TLR in TI-I responses or BCR plus CD40 in TD responses), resulting in appropriately altered patterns of NF-κB activation.

to be unlikely, however, because CD40 stimulation induces Bcl-X_L gene transcription in REL-deficient B cells. An alternative possibility is that REL may be induced for a longer duration than REL-A(p65) in response to BCR crosslinking (Figure 2). The proposed difference in kinetics of REL-A(p65) versus REL expression is based on two considerations. First, analogous to our observations in $CD4^+$ T cells, REL-A(p65) nuclear induction after BCR stimulation is likely to be short-lived because of new IκBα synthesis and IκBα-mediated downregulation. Second, REL induction in both B and T cells is calcineurin dependent (Venkataraman et al., 1995). This part of the REL response is due to new *Rel* gene transcription and translation and requires the slow Ca^{++} influx that is regulated by Btk in mature B cells (Bajpai et al., 2000; Petro et al., 2000). Transcriptional induction of the *Rel* gene in response to the T cell receptor was recently shown to be mediated by the transcription factor NFAT (Grumont et al., 2004). A similar mechanism may apply in BCR-stimulated B cells. As a consequence of de novo *Rel* transcription, higher expression of REL present in activated cells may override IκBα-mediated downregulation. The transient REL-A(p65) response, and more long-term c-REL response, implicates c-REL-containing NF-κB proteins as the major mediators of antiapoptotic gene expression after extended BCR stimulation. Thus, in REL-deficient B cells, NF-κB-dependent survival signals are short lived.

Protracted REL induction in mature B cells may also contribute to the differential sensitivity of immature versus mature B cells to BCR-induced death. Immature B cells do not induce high amounts of REL (Feng et al., 2004), presumably because of diminished *Rel* gene transcription. In the absence of de novo REL protein synthesis, its nuclear induction is likely to be short-lived like that of REL-A(p65), leading to inefficient long-term

transcriptional activation of NF-κB-dependent antiapoptotic genes. Additionally, immature B cells exhibit an imbalance in PI3K and PTEN activity in response to BCR crosslinking, in part through high levels of phospholipid phosphatase PTEN (H.-C. Liou, personal communication). By dephosphorylating PIP3 to PIP2, PTEN reduces Akt kinase activity, which may influence the cellular outcome by elevating proapoptotic pathways such as that involving Bim and by reducing NF-κB induction. The combined effect of accentuated apoptosis and transient NF-κB-dependent antiapoptotic gene expression would make these cells particularly sensitive to BCR crosslinking. In mature B cells the converse combination of increased Akt activity, due to PI3 kinase function, and temporally extended nuclear REL expression, due to Btk and PLCγ function, would favor cell viability over apoptosis.

T-Dependent B Cell Responses

Affinity maturation of the antibody response, and generation of switched antibody isotypes, requires T cell help (McHeyzer-Williams et al., 2006). $CD4^+$ T cells are activated by peptide-MHCII complexes on antigen-presenting cells, costimulatory signals conveyed via CD80 and CD86, and inflammatory cytokines. This occurs in the T cell-enriched regions of the secondary lymphoid organs and leads to clonal selection and proliferation. Concurrently, antigen-specific B cells must be activated in the B cell-rich follicles of the spleen in response to immune complexes bound to follicular dendritic cells (FDC). In this way, costimulatory effects of the CD19-CD21 complex may help to overcome anergy- or apoptosis-inducing effects of the BCR alone. In a crucial next step, antigen-activated B and T cells must come together to initiate T-dependent B cell responses. One route by which the right cells find each other is by B cell-mediated antigen presentation to cognate T cells.

The NF-κB milieu of B cells that receive T cell help differs from that of B cells undergoing T-independent activation primarily because of signaling via CD40. CD40, a cell-surface molecule related to TNF receptors, is constitutively expressed on mature B cells. Its ligand, CD40L (CD154), is also a plasma-membrane protein that is expressed only on activated T cells. Thus, B cells encountering activated T cells in the spleen leads to CD40 signaling that activates both canonical and noncanonical NF-κB pathways persistently (Zarnegar et al., 2004) (Figure 2). The canonical NF-κB pathway mediates B cell survival via induction of antiapoptotic Bcl-2-family genes such as Bcl-X_L. Unlike BCR crosslinking, however, Bcl-X_L induction in response to CD40 does not require REL, presumably because of the extended duration of REL-A(p65) induction. However, REL activated by the canonical pathway remains essential for CD40-dependent proliferation in vitro (Zarnegar et al., 2004). Furthermore, CD40 also activates PI3 kinase, which may attenuate other proapoptotic pathways. The overall result is that CD40 provides long-term survival signals to B cells and induces higher levels of B cell proliferation than are seen with anti-Ig crosslinking alone. It is interesting to note that despite being a relatively strong inducer of the canonical NF-κB pathway, CD40 leads to long-term REL-A(p65) activation. This could be because CD40 signals to IKKβ are enhanced by Akt, whose simultaneous activation (Andjelic et al., 2000; Calderhead et al., 2000) results in increased IκBα turnover and, therefore, less effective IκBα-dependent downregulation of REL-A(p65). Additionally, unlike antigen-receptor- or TNF-R-initiated NF-κB activation pathways, the CD40 signal itself may not be subject to effective feedback inhibition (Moore and Bishop, 2005).

CD40 also upregulates Fas on B cells, making these cells for the first time susceptible to both mitochondria- and death receptor-initiated apoptosis. The importance of the latter pathway is reflected in dysregulation of humoral immunity and autoantibody production in the absence of Fas. Fas-induced death of B cells is antagonized by signals initiated at the BCR (Rothstein et al., 1995); such signals must exceed a threshold to be protective (Foote et al., 1998; Mizuno et al., 2003). This may be one reason why tolerized B cells, which have attenuated BCR signaling, are particularly sensitive to Fas-dependent apoptosis in vivo (Rathmell et al., 1995). Several studies also indicate a role for Fas in suppressing the early generation of antibody-secreting cells in transgenic mice that express autoreactive BCRs (Chen et al., 2006; Culton et al., 2006; William et al., 2006). The ultimate breakthrough from tolerance in these animals may involve mutation of BCRs to higher-affinity somatic variants that effectively protect against Fas-mediated death. *Cflar* (c-FLIP), an NF-κB target gene that inhibits caspase 8 activation in the DISC downstream of Fas, is considered a primary mediator of the protective effects of the BCR (Hennino et al., 2000; Wang et al., 2000). Interestingly, CD40 itself has been proposed to antagonize Fas under some circumstances by inducing another NF-κB-dependent gene, *Gadd45b* (Zazzeroni et al., 2003). Several other mechanisms have been shown to protect B cells from Fas; these include IL-4 initiated signals and inducible proteins such as FAIM and Bcl-X_L (reviewed in Mizuno et al., 2003). The diversity of protective pathways have led to the categorization of B cells as type II with regard to Fas signaling, in which both mitochondria- and caspase-8-initiated death pathways contribute substantially to Fas-dependent apoptosis (Bidere et al., 2006).

Recently, the coreceptor complex associated with BCR signaling was shown to be a means of crossing the protective threshold. Barrington et al. (2005) found that B cells activated by medium-affinity antigens survived in spleen follicles only if they expressed CD21; otherwise, these cells were eliminated via the Fas pathway. In contrast, high-affinity antigen-stimulated B cells did not require CD21 to survive, suggesting that these cells crossed the protective threshold without coreceptor help. Coreceptor-dependent survival was proposed to be mediated by c-FLIP. Because this is a known NF-κB target gene, coreceptor stimulation may increase NF-κB function in activated B cells. This possibility parallels the situation in $CD4^+$ T cells, where costimulation via CD28 increases the amount of NF-κB in comparison with activation by the TCR alone (Kane et al., 2002; Schmitz and Krappmann, 2006). The NF-κB effect in $CD4^+$ T cells is strictly costimulatory in that CD28 alone does not activate this pathway, and it is mediated by PI3 kinase-dependent Akt activation. Narayan et al. (2006) have proposed that activated Akt directly interacts with CARMA1 in the TCR signaling cascade to

increase IKK activity, and thereby NF-κB. Because CD19 also stimulates PI3K and Akt (Otero et al., 2001; Wang et al., 2002), it is possible that Akt synergizes with the BCR-initiated canonical NF-κB pathway to enhance NF-κB activation and NF-κB-dependent gene expression. Moreover, CD19 has been shown to activate Btk (Fujimoto et al., 2002), which is another kinase known to impact BCR-induced NF-κB. Cumulatively, these observations are consistent with the idea that the protective role of the BCR against Fas-initiated cell death is mediated by NF-κB-dependent antiapoptotic gene expression; coreceptor stimulation may accentuate the NF-κB response when BCR signals alone are insufficient. In keeping with the proposed protective role of NF-κB against Fas, a major signaling defect in anergic B cells is reduced NF-κB activation (Jun and Goodnow, 2003).

NF-κB Function in the Germinal Center

Antigen-specific B-T interaction in the T-rich zone of the spleen leads to cell proliferation. One of the choices open to the expanded B cell pool is to terminally differentiate into short-lived plasma cells by activating the transcriptional repressor Blimp-1 (Shapiro-Shelef and Calame, 2004). Another choice is to migrate back into follicles to establish germinal centers (GCs). GCs are highly specialized structures generated as a result of cell-cell and chemokine-based interactions among FDC, B cells, and T cells. Here B cells undergo somatic hypermutation (SHM) and class-switch recombination to produce high-affinity antibodies with the requisite effector function. Both processes are the result of extensive GC B cell proliferation that creates substrates for the mutagenic action of activation-induced deaminase (AID) at the *Ig* loci. Subsequent selection of the highest-affinity variants and death by apoptosis of unselected cells are hallmarks of the GC reaction. CD40-CD40L interactions on B and T cells, respectively, are required continuously to generate and maintain GCs. Additional proliferative vigor is provided by Bcl-6, a transcriptional repressor that is expressed specifically in GC B cells (Fujita et al., 2004; Phan et al., 2005). Bcl-6 is proposed to directly block p53 gene transcription(Phan and Dalla-Favera, 2004), and thereby indirectly downregulate p53-regulated cell-cycle inhibitors. The effects of transgenic Bcl-2 and Bcl-X_L expression on GC cells suggests that cell death in GCs is largely mediated by the mitochondrial pathway. In contrast, Fas probably does not contribute to the bulk of cell death in the GC, but it may help to counterselect unwanted specificities (Mizuno et al., 2003).

Both canonical and non-canonical NF-κB pathways have been implicated in GC function. A role for CD40-induced c-FLIP expression has been suggested as a survival pathway based on the observation that GC cell death in vitro coincides with loss of c-FLIP expression (Hennino et al., 2001; van Eijk et al., 2001). Lack of Bcl-X_L induction via the BCR is proposed to be the basis for short-lived GCs composed of CD45-deficient B cells (Huntington et al., 2006). Thus, consistent with the observations in vitro, survival signals appear to be transmitted via the canonical NF-κB pathway. An important role for the noncanonical pathway is inferred from defective GC formation in BAFF-R- (Rahman et al., 2003; Vora et al., 2003) and p52-deficient (Franzoso et al., 1998) mice, as well as in mice reconstituted with IKKα-deficient cells (Kaisho et al., 2001; Senftleben et al., 2001). Although the noncanonical pathway may also provide survival signals to B cells, its unique role is more likely to be in regulating chemokines and receptors involved in the organization of GC architecture (Bonizzi et al., 2004). It is interesting that activation of this pathway by CD40 does not compensate for BAFF-R-deficiency, suggesting that the requirement for CD40 and BAFF-R may be spatially or temporally segregated. Although these mechanisms operate in B cells, inhibiting the canonical NF-κB pathway in FDC also adversely affects the GC reaction and affinity maturation (Victoratos et al., 2006). The proposed mechanism for this effect is via induction of cell-adhesion molecules that promote B cell viability.

The characteristic features of NF-κB-dependent gene expression may play an important role in the GC. Persistent canonical NF-κB activation via CD40-CD40L interactions is a good candidate for survival and proliferative signals that initiate the GC reaction (Figure 3, arrow "a"). The extent of proliferation may be controlled by attenuation of CD40 signaling, perhaps as a result of limited availability of cognate T cells or negative regulators of CD40 (Aiba et al., 2006; Qian et al., 2004). When CD40 signals ceased, NF-κB and NF-κB-dependent antiapoptotic genes would be downregulated, ultimately leading to cell death (Figure 3, arrow "b"). This is reminiscent of growth-factor-withdrawal-induced $CD4^+$ T cell death after primary clonal expansion (Marrack and Kappler, 2004) and may account for the bulk of cell death in the GC. By analogy, this kind of cell death would proceed by the mitochondrial pathway, for which there is good evidence as described above. Therefore, Bcl-2-family genes, such as Bcl-X_L, would be primary mediators of cell survival. A period of cell viability following each round of proliferation, determined by the kinetics of downregulation of antiapoptotic proteins, would provide the opportunity for AID function and selection.

Renewed BCR signaling, if it occurs within the limited viable phase, has the potential to rescue a fraction of these cells from programmed cell death (Figure 3, arrows "c" and "d"). Such a signal can induce two NF-κB-dependent outcomes via the canonical pathway. First, by reactivating Bcl-X_L (and related antiapoptotic genes), the signal may extend the lifetime of cells and thereby increase the likelihood of their receiving T cell help for another proliferative burst. Second, by activating genes such as those encoding c-FLIP and GADD45β, the BCR may protect cells from Fas-mediated death when they re-encounter $CD4^+$ T cells. Although these genes only temporarily extend cell viability, the dual level of protection thus generated (against cell-intrinsic and cell-extrinsic apoptosis) may play a role in the maturation of the B cell response. Altered patterns of SHM in Bcl-2 transgenic mice is consistent with reduced selective pressure in the absence of cell-intrinsic death (Hande et al., 1998; Kuo et al., 1997). However, affinity maturation does occur in these mice, indicating that other forms of cell death must be overcome if high-avidity BCRs are to be selected. A CD19 mutation that prevents PI3 kinase activation reduces affinity maturation, suggesting a role for the coreceptor in the selection process (Wang and Carter, 2005). In the absence of

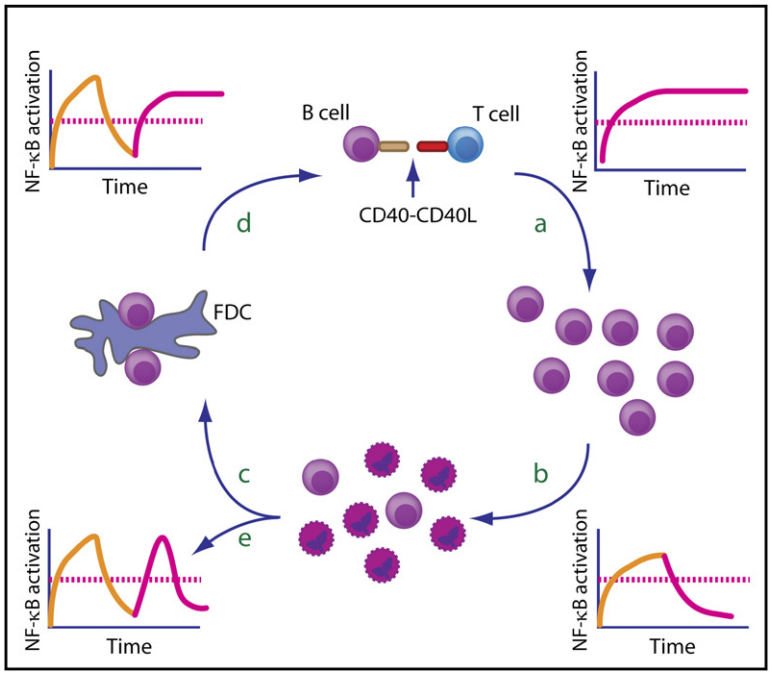

Figure 3. Hypothesized NF-κB-Regulated Survival Signaling during T-dependent Responses

CD40 stimulation induces long-term NF-κB activation via both canonical and noncanonical pathways (arrow labeled "a"). The graph represents canonical NF-κB activation (Y axis = NF-κB amount, X axis = time). A dotted line represents the NF-κB-dependent survival threshold), which is essential for survival and proliferation. Loss of CD40 signals leads to decay of NF-κB, and NF-κB-dependent survival-gene expression (graph next to arrow "b"; a yellow line represents the state of NF-κB activation during the preceding phase; a red line represents NF-κB downregulation that occurs during phase "b"). Cells die (indicated by broken outlines) when NF-κB levels fall below a threshold. The majority of cells generated by proliferation may die at this stage by a cell-intrinsic mechanism. A small proportion of cells that bind antigen on FDC will reactivate NF-κB via the BCR. Two possible NF-κB outcomes of BCR signaling are considered. Recognition of limiting antigen (arrow "d") may activate low-level, persistent NF-κB, which if it exceeds the survival threshold will lengthen the viable phase of these cells (represented in graph next to arrow "d"). As a consequence, the probability of re-encountering T cell help increases. BCR avidity, which determines the level of NF-κB induction, may establish protection against one, or both, cell-death pathways as described in the text. Alternatively, extensive BCR crosslinking (arrow "e") may result in NF-κB downregulation below the survival threshold (represented in graph next to arrow "e"), thereby leading to cell death. In this way, limiting antigen concentration is crucial for an effective response.

CD19 signaling, survivors may be stochastically selected from the expanded pool, and this may lead to the observed somatically mutated, but antigenically unselected, GC population.

The observation that high antigen amounts result in death of antigen-specific GC B cells (Han et al., 1995; Pulendran et al., 1995; Shokat and Goodnow, 1995) suggests that positive selection at this stage is stringently regulated. Organization and immune-complex presentation by the FDC network may play an important role in this process. In addition to expressing NF-κB-dependent cell-adhesion molecules, the FDC may also regulate the level and distribution of antigen for B cell recognition. Limiting antigen concentration allows for rescue of cells that express the highest avidity BCRs and minimizes the chance of extensive BCR crosslinking that may induce apoptosis. If BCR-induced antiapoptotic gene expression plays a role in selection as described above, then a high dose of antigen may trigger cell death by acute, and therefore transient, NF-κB induction (Figure 3, arrow "e"). In the presence of limiting antigen, NF-κB induction may be longer lived, although quantitatively lower. Additionally, strong BCR signals may also activate proapoptotic proteins, such as Bim, that tilt the balance toward cell death.

Fas-initiated cell death may feature two stages during the GC reaction. Human GC cells have been shown to express high levels of Fas and to contain preassembled DISC complex in the cytosol. These cells also express high levels of c-FLIP, presumably induced during CD40-dependent proliferation. Because cell death in culture coincides with loss of c-FLIP, it has been proposed that GC B cells may be susceptible to Fas-initiated death by a cell-intrinsic pathway. This mechanism may also apply in vivo during the decay phase after each round of proliferation. However, its contribution to cell death at this stage is probably not significant or compensated effectively by growth-factor-withdrawal-induced death. Fas may play a more prominent role in selecting the highest-affinity BCR variants prior to subsequent rounds of proliferation, when expression of CD40-induced protective proteins, such as c-FLIP, GADD45β, and Bcl-X_L, is at its lowest. At this stage a range of BCR avidities may provide the initial survival signal against death-by-neglect and thus maximize the likelihood of encountering T cells. However, upon the encounter with FasL- and CD40L-bearing T cells, there may be imposed additional selective pressure from which only cells with the highest-avidity BCRs, which can counter Fas-mediated cell death, will emerge. In other words, although the majority of postreplicative cells die by cell-intrinsic mechanisms, the presence of Fas would accentuate positive selection of the highest-avidity B cells. Conversely, unwanted specificities would emerge in the absence of Fas, as seen in certain experimental circumstances (Takahashi et al., 2001).

Conclusion

B cell homeostasis is governed by cell-intrinsic and cell-extrinsic mechanisms. The importance of proapoptotic and antiapoptotic proteins that affect mitochondrial integrity has been convincingly demonstrated by genetic studies, as has the role of death receptors such as Fas. Both death pathways are regulated by the transcription factor NF-κB via inducible expression of antiapoptotic genes. The goal of this review has been to provide an understanding of the relationship between

the properties of NF-κB and the appropriate control of B cell apoptosis in the periphery. In particular, I have considered the kinetics of NF-κB activation, possible differences between closely related family members REL-A(p65) and REL, the intersection between the canonical and noncanonical pathways of NF-κB activation, and the role of the BCR coreceptor in signaling to NF-κB. Although the present analysis is mostly restricted to responses of a small number of single receptors, the working hypothesis is that the net contribution of the NF-κB pathway in specific circumstances will be governed by the combined patterns of NF-κB activation by individual receptors. Lastly, the cellular milieu in which the action takes place is obviously much more complex than the unabashedly NF-κB-centric views described here; thus, integration of the NF-κB response with other concurrently operative pathways will be required for a complete description of the B cell immune response.

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