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News and Commentary

NF-κB guides the survival and differentiation of developing lymphocytes

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While dysregulated NF-κB contributes to many diseases, including cancer, diabetes, chronic inflammation and autoimmunity and therapeutic strategies have focused on curbing NF-κB's activities; this transcription factor family is also essential for the normal healthy state of the organism. For example, NF-κB activates host defenses against invading pathogens by shaping the response of many cell types, including those of mature lymphocytes responsible for adaptive immunity. This review will discuss how NF-κB also guides the development of these lymphocytes, ensuring their survival at critical stages and assisting in their progression on the correct path to maturity, readying them to respond to foreign pathogens, while preserving self-tolerance.

Developing T and B lymphocytes progress through phenotypically and functionally distinct stages before reaching maturity. As they proceed, they encounter quality control checkpoints. If a developmental program is not properly executed or if autoreactive antigen receptors are acquired, the affected lymphocytes are eliminated. At many checkpoints, death by apoptosis appears to be the default pathway unless the developing lymphocytes receive a survival signal, usually a transient signal that will sustain these cells until the next checkpoint. Successful execution of the assigned developmental program empowers developing lymphocytes to receive transient 'go' signals, and in many cases these signals engage NF-κB in a cell-autonomous manner to 'save' the lymphocytes. Activation of NF-κB counteracts not only death by default but also threats from toxic cytokines. In addition, it helps developing cells to progress along the correct path. We will focus on particular developmental checkpoints and discuss recent findings that highlight the complex roles of NF-κB in lymphocyte survival and developmental progression. Figures 1 and 2 highlight the contributions of NF-κB to B and T lymphocyte development, respectively.

Early Precursors

Early lymphocyte precursors may need the cell-autonomous functions of NF-κB to protect them from cytotoxic factors encountered during development, specifically TNFα. RelA-deficient lymphocyte precursors derived from fetal liver had a reduced capacity to regenerate lymphocytes when adoptively transferred into lethally irradiated hosts, unless they also lacked the TNF receptor I.[1] Furthermore, *ex vivo* B-cell cultures derived from the mutant precursors exhibited reduced levels of the antiapoptotic regulators, Flip and Bcl-2, and were highly sensitive to the cytotoxic effects of TNFα if this cytokine was added to the culture (see Figure 3). Lymphocyte precursors derived from fetal livers of mice with a greater impairment of NF-κB, as in knockout of both RelA and NF-κB1,[2] or RelA and c-Rel,[3] or IκB kinase (IKK)β,[4] were unable to generate lymphocytes following their adoptive transfer into lethally irradiated hosts, but this block was relieved in the presence of wild-type hematopoietic cells or in the absence of TNF receptor I signaling. The partial or complete block in lymphopoiesis of the various NF-κB-impaired precursors was apparently due to high levels of cytotoxic TNFα generated via an unknown mechanism by mutant non-lymphoid hematopoietic cells.[1–5] In addition, however, the block was also likely due to the inability of the mutant precursor lymphocytes to protect themselves from TNFα, since they lack sufficient NF-κB activity to overcome apoptotic pathways via NF-κB-induced survival functions (see Figure 3). The near-total block of NF-κB observed in NEMO (IKKγ) knockout mice, in which all IKK-mediated activation of NF-κB is lost, may have prevented all lymphopoiesis even if only normal (low) levels of TNFα were encountered, since the presence of wild-type hematopoietic cells failed to rescue the appearance of peripheral mutant lymphocytes.[6,7] However, this hypothesis remains unproven.

The Pre-antigen Receptor Stage

Successful rearrangement of T-cell receptor (TCR)β or Ig μ-heavy chain and combination with a surrogate TCRα or surrogate Ig light chain leads to the expression of a pre-TCR or a pre-B-cell receptor (BCR) on the surface of developing T cells (thymocytes) or B cells, respectively (Figures 1 and 2). If the generation of pre-antigen receptors is unsuccessful, the affected cells are eventually eliminated by default. If the developing cells do express a functional pre-antigen receptor, then tonic (ligand-independent) signaling is critical for their survival[8–11] (Figure 3). Tonic signaling of the pre-antigen receptor signal is essential for survival at least in part because developing (murine) B cells and developing thymocytes become less responsive to IL-7, which functions as a survival signal prior to expression of the pre-antigen receptor.[12] There is ample evidence (see below) that pre-antigen receptor-mediated activation of NF-κB is important for cell survival and thus developmental progression.[8–11] Although this review will not discuss mechanisms by which antigen receptor signals to NF-κB, it is worth noting that pre-antigen receptor signaling (as well as antigen receptor signaling by later-stage thymocytes and immature B cells) differs in some details from the

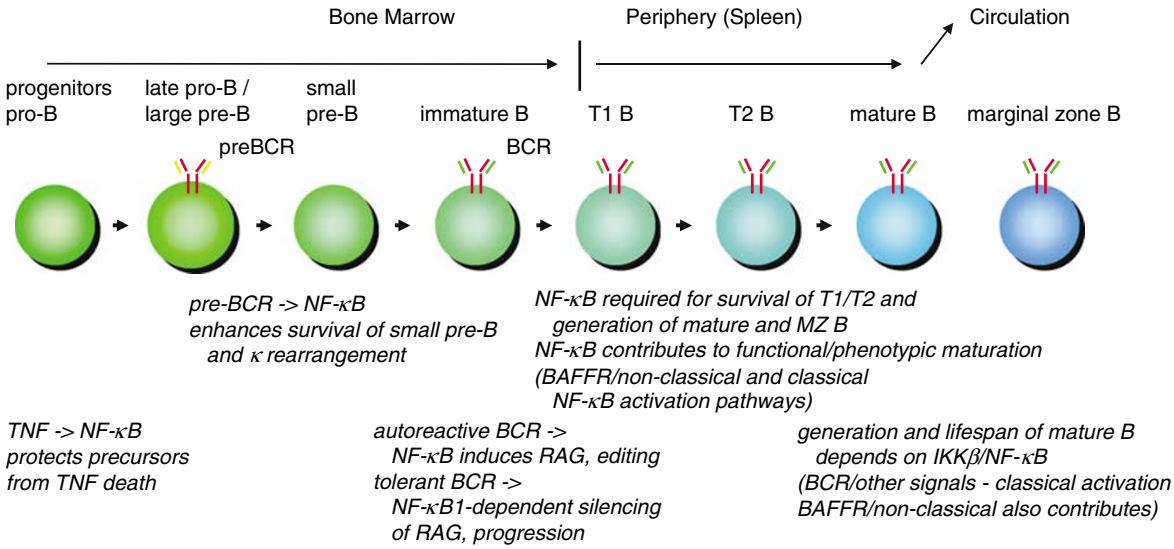

Figure 1 NF-κB in B2 B-cell development. An abbreviated schematic representation of B2 B-cell development, highlighting some of the contributions of NF-κB at various developmental checkpoints. T1, Transitional 1; T2, Transitional 2. See text for a detailed discussion

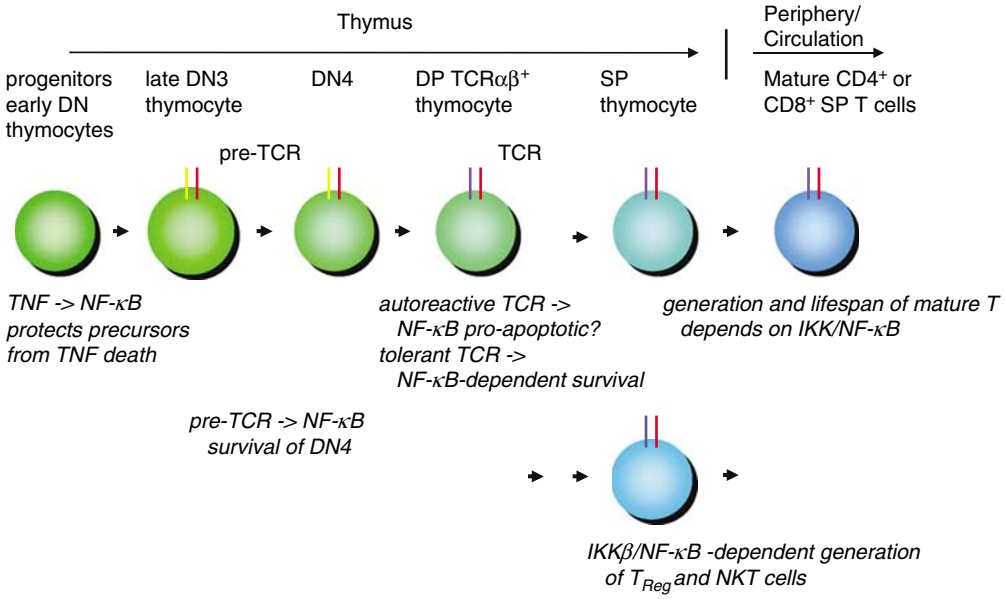

Figure 2 NF-κB in T-cell development. An abbreviated schematic representation of T-cell development, highlighting some of the contributions of NF-κB at various developmental checkpoints. DP, double-positive; DN3, DN4, double-negative stages 3, 4; SP, single-positive. T_{Reg}, T regulatory cell; NKT, Natural Killer T cell. T_{Reg} and NKT cells branch off at some point after TCR expression on thymocytes. See text for detailed discussion

antigen receptor signaling of mature cells. For example, while mature T and B cells require PKCθ and PKCβ, respectively, to activate NF-κB via their antigen receptors, their immature stages do not.[13]

Blocking NF-κB activation in pre-TCR-positive cells with the IκB super-repressor led to apoptosis *ex vivo*.[8] Pre-TCR-mediated activation of NF-κB induced the antiapoptotic Bcl-2 family member, A1, which is necessary for survival.[9] Similarly, pre-BCR-mediated signaling activated NF-κB,[10] and blocking this activation with the IκB super-repressor impaired progression, reducing the population of cells at stages after the pre-BCR$^+$ stage.[10,11] Finally, ectopic expression of Bcl-xL largely overcame the survival defect and allowed cells to progress.[11]

Generation of Immature B Cells

NF-κB has been implicated in the Ig light chain gene rearrangements of small pre-B cells (Figure 1). It may control the demethylation of the κ locus necessary to make this locus fully accessible for germline transcription and DNA rearrangements.[14] The κ intronic enhancer κB-binding site and NF-κB

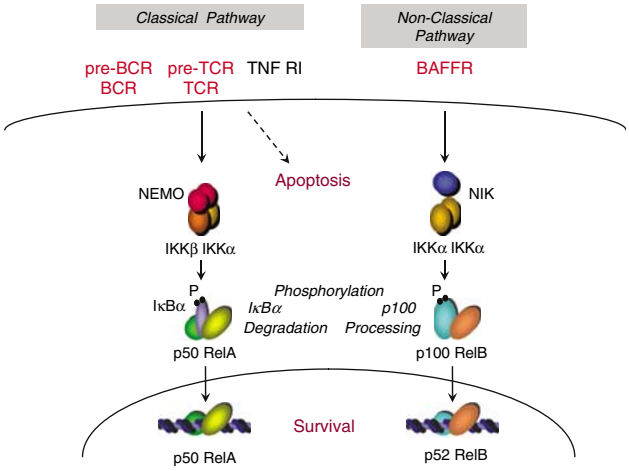

Figure 3 The classical and nonclassical NF-κB activation pathways. A simplified schema depicting critical components of the classical and the nonclassical (alternative) pathways for activation of NF-κB. In addition to promoting survival via NF-κB target genes, the TNF receptor I (TNF RI) also stimulates competing apoptotic pathways. B- and T-cell antigen receptors (BCR, TCR) may in some contexts enhance apoptotic pathways, but usually they contribute to survival. BAFF receptor (BAFFR) signaling has only been associated with survival functions. The classical pathway depends critically on IKKβ and NEMO (IKKγ), while the nonclassical pathway requires NF-κB-inducing kinase (NIK) (as well as IKKα). p50, RelA, p100, and RelB are members of the Rel/NF-κB family of polypeptides. p50 is derived from p105 (NF-κB1) and p52 is derived by processing from p100 (NF-κB2). See text for a detailed discussion

activity were shown to be involved in mediating demethylation of a κDNA segment in transfection experiments *in vitro*.[14] However, this particular κB-binding site was not essential for rearrangement *in vivo*.[15] This does not rule out a redundant role for this site nor does it exclude a contribution of NF-κB at other sites. The most notable experiment supporting a role for NF-κB in demethylation utilized v-Abl-transformed pre-B-like cells. These transformed cells are partially impaired in NF-κB activation and in light chain gene rearrangement. Transfection of RelB into these cells induced demethylation of the κ locus.[14] Prior evidence had already implicated the impaired activation of NF-κB in the v-Abl-transformed cells as critical to the block of germline transcription and light chain gene rearrangement, since this block was relieved provided NF-κB was activated, for example, with lipopolysaccharide or by inactivation of a temperature-sensitive v-Abl mutant.[16]

Selection of Immature B Cells

A recent intriguing insight into roles of NF-κB during development was revealed by investigations of immature B-cell selection.[17] Immature B cells are the first stage at which developing B cells express a BCR (Figure 1). If a given BCR is autoreactive, the cell bearing it is either eliminated by apoptosis or reactivates RAG recombinase, so that it can 'edit' (further rearrange) the light chain to generate a different BCR. The surprising discovery was made that RAG gene expression is negatively regulated by NF-κB1 and positively regulated by RelA- and c-Rel-containing NF-κB dimmers.[17] These results suggest that weak/tonic signaling via the BCR may provide a positive selection signal that actively suppresses RAG expression (possibly via p50/NF-κB1 homodimers) thus preventing further rearrangement. p50 homodimers have previously been implicated in gene repression.[18,19] Conversely, a strong autoreactive signal may induce RAG expression and thus editing via activation of dimers containing RelA and c-Rel. This highlights an interesting concept that has also emerged in other contexts, namely, different NF-κB complexes may have different or even opposing effects on gene regulation, and it is the ratio of the various complexes that is critical. It will be important to investigate the signaling mechanisms by which the ratios of NF-κB complexes are regulated. It is also important to note that elimination of a given NF-κB protein may have different consequences in different contexts, depending on which of its associated dimer combinations is most physiologically important.

The WEHI231 immature-like B-cell line (and similar lines) may provide an *in vitro* model for selection against autoreactive cells. When stimulated through the BCR, these cells exhibited an initial transient activation of NF-κB, then later, apoptosed.[20] Apoptosis could be prevented by a more sustained activation of NF-κB, for example, via CD40 stimulation.[20] It is not known whether WEHI231 mimics normal B-cell selection, but it suggests the possibility that autoreactive BCR-mediated activation of NF-κB *in vivo* is similarly transient. Cells so instructed to edit their receptors may then be on borrowed time, if BCR-mediated NF-κB activation is short-lived. If that is so, then these cells may be highly dependent on other survival factors that may extend the time available for receptor editing. Possible survival factors for pre-B/immature B cells may include BAFF acting via BAFF receptor (BAFFR),[21,22] hemokinin-1,[23] and thymic stromal lymphopoietin.[24] BAFFR signals principally through NF-κB that is activated by the nonclassical (alternative) pathway that processes NF-κB2 p100 to p52[22] (see below and Figure 3).

Positive and Negative Selection of T Cells

Having successfully rearranged their TCRα chains, TCR$\alpha\beta$ double-positive (DP) thymocytes are subject to positive and negative selection (Figure 2). Thymocytes bearing autoreactive TCRs are eliminated by apoptosis, as are those unable to recognize MHC. Those that weakly recognize self-antigens in the context of a matched MHC are positively selected. Some of the roles proposed for NF-κB in this process are surprisingly counterintuitive.[25–27] Inhibition of NF-κB has the paradoxical effect of blocking negative selection, suggesting that, here, NF-κB has a proapoptotic function (Figure 3). This conclusion was reached in two different model systems. Firstly, transgenic mice with the IκB super-repressor[25] or a dominant negative IKKβ[26] in their T cells were challenged with anti-CD3 antibodies *in vivo* (to mimic autoreactivity). In a second, more physiological model, mice were generated bearing T cells that expressed both the IκB super-repressor and an autoreactive TCR transgene.[27] In an analogous model for positive selection (a TCR transgene that weakly recognized self-antigens in the context of an appropriately matched MHC), NF-κB activity appeared to have its conventional antiapopto-

tic/survival role as demonstrated by IκB super-repressor impairment of thymocyte progression.[27] It is possible that the NF-κB signal serves, at least in part, to enable signal strength to be assessed by the cell. Impairment of NF-κB activity might be sensed by autoreactive cells as a weak TCR signal, resulting in positive rather than negative selection. By a similar argument, impairment of NF-κB activity under positive selection conditions might be interpreted as a nonexistent signal, prompting death by neglect. Uncertainties remain, for example, negative selection has also been ascribed to active repression of NF-κB, consistent with a positive role for NF-κB in survival. Antigens that cause negative selection were reported to induce expression of an IκB super-repressor-like protein ($I\kappa B_{NS}$) in DP thymocytes.[28] This appears to contradict a proapoptotic role of NF-κB in negative selection, although $I\kappa B_{NS}$ could conceivably be induced by NF-κB itself.

Natural killer T (NKT) and $CD4^+CD25^+$ regulatory T cells (T_{reg}) are positively selected by recognition of self-antigens at the double-positive (DP) stage[29–32] (or, are not negatively selected;[33] Figure 2). Both subsets are highly dependent on NF-κB for their development. Conditional loss of the IKKβ subunit in mice impairs their generation in the thymus,[29,30] as judged by the substantial counterselection of these subsets observed, that is, a disproportionately high number of cells in these two populations still carried and expressed the wild-type IKKβ allele, suggesting a preferential loss of those cells that had deleted this allele (the conditional Cre-mediated deletion is an incomplete and ongoing process during developmental progression of T cells). Other developing thymocyte subsets (the majority) were unaffected by loss of IKKβ (see also below). NKT[31] and T_{Reg}[32] cell development was also partially blocked in other models in which T-cell NF-κB activity was impaired. Although a TCR signaling path like that of mature T cells appears to activate NF-κB in T_{Reg} thymocytes, developing thymic NKT cells either employ a somewhat different TCR signaling path, independent of Bcl-10, or utilize other signals.[30] In addition to the intrinsic role of NF-κB in NKT development, the development of these cells requires an intact RelB in stromal cells.[31]

Single-positive Thymocytes and Homeostasis of Peripheral T Cells

Once TCRαβ DP thymocytes have been positively selected, they progress to CD4 or CD8 single-positive (SP) thymocytes, which soon leave the thymus to enter the periphery as mature, naïve T cells (Figure 2). Evidence suggests that starting before exit from the thymus and continuing in the periphery, T-cell survival requires a low level of NF-κB activity. When NF-kB activation by the classical pathway was eliminated in T cells by conditional loss of NEMO (IKKγ) or conditional replacement of wild-type IKKβ with a dominant negative mutant form, the peripheral T-cell population was depleted. This was shown by the counterselection of cells that had undergone Cre-mediated recombination, leaving only those cells that still carried and expressed the wild-type IKKγ/IKKβ allele (conditional loss of IKKγ or introduction of dominant-negative IKKβ is ongoing and not complete; see previous discussion).[29] These studies also concluded that loss of IKK activity that occurred after T cells had matured severely reduces their life expectancy.[29] In addition, counterselection was already apparent earlier in development, in thymocytes, especially in the CD8+ population. That late-stage thymocytes already require NF-kB activity for survival had been foreshadowed in prior studies with partially inhibitory IκB super-repressors.[34]

Splenic Transitional B Cells

After the late immature (early transitional) stage, B cells leave the bone marrow. They enter the spleen, where they undergo several functional and phenotypic changes before maturing into antigen-responsive B2 B cells. They enter the spleen as transitional 1 (T1) B cells, and then progress through at least one more stage, T2, before finally becoming mature follicular B (B2) cells that enter the peripheral circulation[22] (Figure 1).

At some point during the transitional stages, some B cells are diverted to become resident splenic marginal zone (MZ) B cells. MZ B cells respond rapidly to a more limited set of antigens, such as repeat structures present on surfaces of pathogens, and they do not require any T-cell help. The generation of MZ B cells is adversely affected by marginal impairment of NF-κB activity, such as loss of single NF-κB factors, for example, NF-κB1[35] or RelB,[36] which has only small effects on the follicular B-cell population. However, some level of NF-κB activity is needed for all B2 B cells to pass through the transitional phases. B-cell development was completely blocked at or just after the T1 to T2 transition in compound knockouts of NF-κB1 and NF-κB2[22] or c-Rel and RelA,[5] respectively. NF-κB has an essential cell-autonomous survival function during the T1/T2 progression. Absence of the afore-mentioned factors correlated with reduced expression of the antiapoptotic regulators Bcl-2 and A1 and increased spontaneous apoptosis of transitional B cells.[5,22] Furthermore, ectopic expression of Bcl-2 rescued mutant B cells of both compound knockouts from elimination at the T1/T2 boundary[5] (EC and US, unpublished). However, these cells still failed to fully mature. Thus, in addition to its essential survival functions, NF-κB also contributes to the final maturation program in B cells, including their ability to respond positively to antigens and secrete antibodies.

The complete block in developmental progression caused by the loss of NF-κB1 and NF-κB2 suggests contributions from both the classical (via NF-κB1) and the nonclassical/alternative activation path (via NF-κB2) at this stage of development (Figure 3). The two pathways may have partially redundant functions in B-cell survival. Results with BAFF and BAFFR knockouts imply that this signaling pair is responsible for activating the alternative pathway in developing B cells,[22,37] but the identity of the signal(s) which activate the classical pathway is, as yet, unknown. As cells progress into and through the T2 stage, the mature BCR signaling machinery may provide a positive survival signal via the classical pathway (Figure 3).

Compound loss of NF-κB1 and NF-κB2 in B cells also blocked development of B1 B cells (EC and US, unpublished). B1 cells are thought to constitute a self-renewing population in adults originating directly from fetal precursors. Little is known

about B1 B development and the role of NF-κB is not yet understood. While the BCR could be responsible for activating classical pathway-mediated activation of NF-κB in these cells (see Figure 3), given that the more limited repertoire of B1 BCRs often responds to self-antigens which would signal via the classical pathway, the signal for activating the alternative pathway is unclear since loss of BAFF/BAFFR did not affect this population.[37]

Homeostasis of Mature B Cells

From the late transitional stage of B-cell development through to and including the mature stage, NF-κB appears to be required for long-term survival. Conditional loss of IKKβ in B cells (some IKK activity remained) eliminated all mature B cells (the few remaining cells still expressed IKKβ),[38,39] just as loss of the BCR did,[40] suggesting that classical activation of NF-κB via a tonic BCR signal is needed for extended cell survival (Figure 1). Also, BAFF-mediated activation of NF-κB via the nonclassical pathway contributes to long-term maintenance of B cells (Figure 3). Loss of elements of this pathway, including IKKα, decreased the B-cell population in the periphery and increased turnover.[41,42] Finally, conditional loss of NEMO or replacement of IKKβ with a dominant negative version resulted in counterselection of mutant B cells beginning during the transitional phase, with the survivors yet to undergo Cre-mediated deletion (as for T cells, see above).[38] This is consistent with the results of the compound knockouts in transitional B cells described in the previous section.

Perspectives

In addition to its direction of many of the responses of both mature T and B lymphocytes to a pathogenic challenge, which we have not discussed here, NF-κB occupies a central position in the development of these lymphocytes. The need for NF-κB's intrinsic and cell-autonomous functions is apparent very early in lymphocyte development, is critical as cells pass through various checkpoints, and persists in maturity, where NF-κB activity is essential for an extended lifespan, independent of antigenic challenge. While the threshold level of NF-κB that is required early may be minimal and redundantly provided by the various NF-κB factors, later checkpoints appear to require a higher threshold level of NF-κB activation and/or are more dependent on specific factors. Although cell survival is the most visible function of NF-κB during development, recognition of its 'differentiation'-related functions is growing, including functions important for the selection of acceptable antigen receptors in both B and T cells. In B cells, the latter functions include modulation of RAG gene expression and in T cells they appear, paradoxically, to include apoptosis-promoting activities during negative selection, contrasting with its usual promotion of survival. Many targets of NF-κB at the various checkpoints in development remain unknown, but in the case of cell survival functions they include antiapoptotic Bcl-2 family members, some of which can be directly controlled by NF-κB, including Bcl-xL and A1. It is likely that the concerted actions of multiple targets of the various NF-κB dimers are needed to guide lymphocytes along the correct developmental path and through the checkpoints that decide life or death.

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