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REVIEW

The complicated role of NF-κB in T-cell selection

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The nuclear factor (NF)-κB transcription factor family plays important roles in the immune system. Aberrant NF-κB signaling is frequently associated with inflammation and autoimmune diseases but the underlying mechanisms are not fully understood. Recent studies show that NF-κB plays a critical role in T-cell central tolerance. Two NF-κB signaling pathways have been identified: the canonical pathway and the alternative pathway. In the establishment of T-cell central tolerance, the alternative pathway appears to be the key signaling component in thymic stromal cells for their development and function, while the canonical pathway exerts its function more in autonomous T-cell selection. This review intends to summarize the current understanding of the role of NF-κB in establishing T-cell central tolerance and highlight unsolved intriguing questions for future work.
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INTRODUCTION

Nuclear factor (NF)-κB is a family of structurally-related transcription factors. Since its discovery by David Baltimore in 1986,[1] NF-κB has been found in almost all animal-cell types and involved in various cellular processes such as cell growth, apoptosis, differentiation, transformation and response to stimuli. Its role is indicated in numerous physiological and pathological processes ranging from innate and adaptive immune response, inflammation, tumor, organogenesis and development, to synaptic plasticity and memory. This review will focus on the role of NF-κB in the establishment and maintenance of T-cell central tolerance, a critical mechanism forestalling autoimmunity.

NF-κB

Five NF-κB members have been found in mammals: NF-κB1 (p105/50), NF-κB2 (p100/52), RelA (p65), RelB and c-Rel. All NF-κB proteins share a Rel homology domain in their N-termini, which is required for dimerization, nuclear translocation and DNA binding. Only RelA, RelB and c-Rel have a transactivation domain in their C-termini. The other two NF-κB proteins, NF-κB1 and NF-κB2, lack a transactivation domain but instead contain seven ankyrin repeats, a characteristic motif of inhibitor of NF-κB (IκB) that mediates protein–protein interactions. Although RelA, RelB and c-Rel are expressed as their mature forms, NF-κB1 and NF-κB2 are generated as precursor proteins, p105 and p100, respectively. The ankyrin domain is proteolytically cleaved and degraded for generation of the mature forms of NF-κB1 (p50) and NF-κB2 (p52). The five NF-κB proteins can form 15 transcription factors through homo- or heterodimerization.[2] In resting cells, NF-κB dimers are retained in the cytoplasm by IκB proteins. NF-κB dimers are liberated by either degradation of IκB in the canonical pathway, or processing of p100 in the alternative pathway. RelA:p50 and c-Rel:p50 are the major NF-κB dimers activated by the canonical pathway. Processing of p100 to p52 produces and activates the RelB:p52 heterodimer in alternative pathway.

The canonical pathway can be rapidly and transiently activated by a variety of stimuli such as Toll-like receptor ligands, tumor-necrosis factor-α and IL-1, T-cell antigen receptor (TCR) and B-cell antigen receptor (BCR) agonists. Upon stimulation, IκB kinase (IKK) is activated and phosphorylates specific serines in the IκB proteins, leading to their ubiquitination and proteosomal degradation, thus allowing the liberation and nuclear translocation of NF-κB dimers to induce gene expression. Phosphorylatoin of IκBs is predominantly mediated by IKKβ. The ankyrin repeats containing NF-κB1 (p105) and NF-κB2 (p100) can also function as IκB-like proteins to retain NF-κB in the cytoplasm. While processing of p105 is constitutive, p100 processing is regulatory as discussed below.

The non-canonical (or alternative) pathway is usually triggered by non-inflammatory stimuli, such as lymphotoxin-β signaling, CD40L, receptor activator of NF-κB ligand and B-cell-activating factor of the tumor-necrosis factor family. Activation of the alternative NF-κB pathway is strictly dependent on NF-κB-inducing kinase and IKKα, and independent of IKKβ and IKKγ.[3,4] Since the Rel homology domain of p100 is most commonly associated with RelB, activation of this alternative pathway results in nuclear translocation of p52-RelB heterodimer. The alternative NF-κB pathway has been shown to play a central role in the expression of genes involved in development, maintenance and function of primary and secondary lymphoid organs.[5]

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The canonical and alternative NF-κB pathways do not exist in isolation and the close cross-talk contributes to fine-tune signaling processes through several mechanisms: (i) canonical NF-κB pathway regulates p100 and RelB expression and homeostasis;[6–8] (ii) RelA and RelB can form complex, which dampen their individual transactivation activity;[9–11] and (iii) competition for dimerization partners. For example, absence of NF-κB1/p50 leads to elevated levels of RelA:p52 and constitutive processing of p100.[8] Similarly, increased p50 levels are found in *Nfkb2*$^{-/-}$ murine embryo fibroblasts, leading to formation of RelB:p50 dimers.

CENTRAL TOLERANCE

Central tolerance is the primary mechanism in shaping the repertoire of lymphocytes in early life to prevent autoimmunity. This occurs in the bone marrow for developing B cells and in the thymus for developing thymocytes. In this review, we will focus on the role of NF-κB in T-cell central tolerance. We will discuss several aspects that NF-κB is involved in control of T-cell central tolerance: (i) medulary thymic epithelial cell (mTEC) development and function; (ii) regulatory T cell (Treg) development; and (iii) thymocyte negative selection

Alternative NF-κB pathway and mTECs development and function

The thymic stroma fosters the growth, differentiation, and positive and negative selection of the T-cell receptor repertoire. A proper thymic stroma microenvironment is necessary for the establishment of both central tolerance and immune competency. The thymic stromal compartment is composed of several distinct populations of cells which include epithelium, fibroblasts, endothelium, macrophages and dendritic cells, each of which has a distinct role in T-cell development. Among these populations, mTECs have been the most extensively investigated and have been shown to play a critical role in central tolerance. Importantly, NF-κB has been found to be critically involved in the regulation of mTECs development and function.

mTEC and central tolerance. The thymic medulla has gained consensus to be the main site for both negative selection of autoreactive thymosytes and positive selection of Tregs, two important central tolerance mechanisms. In agreement with the important role of NF-κB in central tolerance, autoimmune phenotype is observed in various mutant mouse strains that have impaired medullary characteristics: *Relb*$^{-/-}$ mice,[12] *aly*/*aly* mice;[13] *Ikka*$^{-/-}$ embryonic thymi grafted nude mice,[14] *Traf6*$^{-/-}$ mice,[15] *Nfkb2*$^{-/-}$ mice,[16,17] *Ltbr*$^{-/-}$ mice[18,19] and *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mice.[20] Mice with these mutations have reduced and disorganized mTECs. Furthermore, the degree of thymic medulla defect seems to be correlated with the severity of autoimmune phenotype: from the mild in *Nfkb2*$^{-/-}$ and *Ltbr*$^{-/-}$ mice to the severe in *Relb*$^{-/-}$, *aly*/*aly* and *Traf6*$^{-/-}$ mice. It needs to be clarified that all of these factors could have multiple effects on both non-hematopoietic cells and hematopoietic cells, which complicates the explanation of the autoimmune phenotype found in these mice. Using advanced techniques such as thymic stroma transplantation and bone marrow transplantation, researchers have largely located the essential role of these molecules in the thymic stromal compartment. It is of note that all of these molecules are involved in the alternative NF-κB pathway, strongly arguing for the critical role of alternative NF-κB pathway in mTECs development, homeostasis and organization and central tolerance.

mTEC development, homeostasis and organization. Given the importance of mTEC in central tolerance, its development, homeostasis and organization becomes an attractive topic in the field of central tolerance. Although the literature suggests an important role of the alternative NF-κB pathway in the regulation of mTEC development, homeostasis and organization,[12,13] it remains largely unclear exactly how the alternative NF-κB pathway regulates generation of mTEC progenitor cells, mTECs differentiation, proliferation, homeostasis and organization into functionally competent medulla.

NF-κB preferentially regulates mTEC rather than cortical TEC (cTEC). Although the mTECs developmental defect is common in various mutant mice bearing mutation/deficiency of NF-κB pathway-related molecules, cTEC development seems largely unaltered. This suggests that NF-κB does not regulate the generation of the common TEC progenitor cells. Instead, it may regulate the branching of the common TEC progenitor to cTEC and mTEC progenitor cells, or at a later stage of mTECs' proliferation, survival and maturation. Interestingly, *Relb*$^{-/-}$ mice lack thymic medulla while cTEC is largely retained. The preferential regulation of mTEC by RelB is also consistent with its higher expression in medulla than in cortex. *Relb*$^{-/-}$ mice may serve as a useful model to investigate how mTEC progenitor cells are differentiated, proliferated and maintained homeostasis.

Some subset of mTECs has been recently revealed to be regulated by NF-κB. Claudin-3^{+}Claudin-4^{+} mTECs defines in adult mice an unique subset of mTECs expressing autoimmune regulator (Aire), which controls thymic expression of a large bunch of tissue restricted antigens (TRAs) and thus plays an crucial role in the induction of T-cell central tolerance toward TRAs.[21] The scientists found that the generation of this unique mTEC population is unaffected in in *aly*/*aly* or *Traf6*$^{-/-}$ mice at embryonic day 11.5.[22] However, this population is profoundly diminished at embryonic day 16.5 in those mice.[22] Significant higher RelB expression is also found in mTECs positive for Claudin-3 and -4.[22] These data suggest that the differentiation and/or proliferation/survival of this crucial mTEC lineage are dependent on RelB activation mediated by NF-κB-inducing kinase and tumor-necrosis factor receptor-associated factor 6. Whether the mTEC differentiation and/or proliferation/survival in the adult are also regulated by the same signaling pathway remains unknown.

NF-κB appears to also participate in the maturation of mTECs. mTECs is heterogeneous based on the expression levels of major histocompatibility complex class II, CD80 and a binding site for lectin Ulex europaeus agglutinin 1 (UEA1). Increasing level of these surface molecules indicates a progressive maturation of mTECs, which is correlated with the function of mTECs.[23] Both lymphotoxin-β receptor (LTβR) and receptor activator of NF-κB (RANK) predominantly activate alternative NF-κB pathway. It was found that *Ltbr*$^{-/-}$ thymi severely lack fully matured UEA1-positive mTECs, while immature mTECs largely remain.[24,25] RANK signaling was also shown to be essential for promoting the maturation of mTECs from CD80^{-} to CD80^{+}.[26–28]

In addition to the development and homeostasis of mTECs, NF-κB might regulate organization of mTEC. In agreement with this, *Ltbr*$^{-/-}$ and *Nfkb2*$^{-/-}$ thymi have disorganized mTEC structure with dispersed small mTEC areas in stark contrast to the connective large mTEC area in wild-type thymi (unpublished data). Similar phenotype was noticed in *plt*/*plt* mice, in which CCR7 ligands (secondary lymphoid tissue chemokine and Epstein–Barr virus-induced molecule 1 ligand chemokine) are deficient. It is completely unknown how NF-κB and CCR7 signaling control mTEC organization; it is also unknown whether and how the disorganized mTECs would impair central tolerance.

It must be noted that the alternative and canonical NF-κB pathways could have a redundant role in mTECs development, homeostasis or organization. In agreement with this, *Nfkb1*$^{-/-}$*Nfkb2*$^{-/-}$ mice have a much more severe mTEC defect than *Nfkb2*$^{-/-}$ mice,[16,29] while *Nfkb1*$^{-/-}$ mice show a normal thymus (our unpublished observation). However, this explanation could be complicated by the facts that NF-κB also plays a role in thymocytes development autonomously (see below) and that cross-talk between thymocytes and the stroma is critical for stromal compartment development. Although *Nfkb1*$^{-/-}$*Nfkb2*$^{-/-}$ bone marrow reconstitution in *Rag1*$^{-/-}$ mice rescued some of the defects found in straight double knockout mice, it is not known whether the mTECs defect is corrected.[29] Thus, it is still not clear whether the canonical and alternative NF-κB pathways have a redundant role intrinsic to mTECs.

mTEC induces negative selection. One important function of mTECs is to present TRAs to developing thymocytes to induce negative selection of organ-specific autoreactive thymocytes.[30] The thymic expression of TRAs is controlled by both Aire-dependent and -independent pathways.[19,21,23] Reduced thymic expression of Aire/TRAs is a common defect found in all of the mutant mice mentioned above. This could be due to both cell-autonomous and developmental effects (reduced mTEC number).

Our data indicate that the alternative NF-κB pathway could regulate Aire/TRA expression through both cell-autonomous and cell-extrinsic mechanisms. Although the mTEC population is partially reduced in *Nfkb2*$^{-/-}$ mice, Aire/TRAs expression is also reduced in *Nfkb2*$^{-/-}$ mTECs on a per cell basis.[16] These data alone do not necessary mean that NF-κB2 pathway regulate Aire/TRAs intrinsically given the possibility that function of thymocytes, therefore T-mTEC cross-talk, could be altered due to NF-κB2 deficiency. However, our data further demonstrate that LTβR agonistic antibody treatment, which can activate the NF-κB2 signaling pathway, also upregulates Aire/TRAs expression, and this effect can be abolished when NF-κB2 pathway is ablated. Thus, these data together suggest that the NF-κB2 pathway can regulate Aire/TRAs in a cell-autonomous manner.

Recently, RANK signaling from 'lymphoid tissue inducer' cells has also been found essential for Aire/TRAs expression in mTECs autonomously.[28] Treatment of 2-deoxyguanosine-terated fetal thymus organ cultures with either anti-RANK antibodies or RANK ligand induces the emergence of CD80^{+}Aire^{+} mTECs from CD80^{-}Aire^{-} mTECs within 2 days. Given the short-term period and significant population of CD80^{+}Aire^{+} mTECs, it is likely that RANK signaling promotes the maturation of CD80^{-} mTECs to CD80^{+} mTECs and turns on Aire/TRAs gene expression. It is unclear whether the role of RANK in promoting Aire/TRAs expression is mediated by NF-κB2 pathway or not. It is also unclear whether and how LTβR and RANK pathway coordinate for thymic Aire/TRAs expression. Furthermore, exactly how NF-κB regulates thymic Aire/TRAs cell-autonomously remains an interesting question. Since no known NF-κB binding sites are found on the promoter of the Aire gene,[31] this indicates that NF-κB could regulate Aire gene expression through other transcriptional factors or epigenetic modification.

mTEC induces Treg generation. CD4^{+}Foxp3^{+} natural regulatory T cells (nTregs) arise in the thymus. Immunofluoresence staining of thymic sections shows that Foxp3 is predominantly expressed in the thymic medulla, suggesting a critical role of the medulla in CD4^{+}Foxp3^{+} Treg development.[32] Consistently, mice with severe defects of thymic medulla, such as *Relb*$^{-/-}$, *aly*/*aly* and *Traf6*$^{-/-}$ mice, all have dramatically reduced numbers of Foxp3^{+} nTregs in thymi (our unpublished finding).[13,15] The underlying mechanism for thymic medulla to control Foxp3^{+} nTreg development is still vastly unknown; however, several possibilities exist: firstly, TRAs could be one of the signals provided by the medulla for nTreg development. A recent study has demonstrated that TRA-specific Tregs can be selected by Aire^{+} mTECs, while the cross-presentation by thymic DCs is not required.[33] However, Aire-deficient mice were found to have normal Treg compartment although thymic expression of some TRAs is reduced.[34] It remains unclear whether single TRA-specific Treg generation is reduced in *Aire*$^{-/-}$ mice. In other words, it is still unclear whether a single TRA is essential for the generation of Treg specific to this particular TRA. Secondly, costimulatory and adhesion molecules expressed on thymic stromal cells are other sets of signals influencing nTreg development in the thymus. For example, deficiency in CD28, CD40, B7 or leukocyte function antigen-1 results in a substantial reduction in Treg cell numbers in the thymus.[35] However, given that the Foxp3^{+} thymocytes become detectable in the late double-positive (DP) stage, it is in controversy where and how the thymic stroma delivers and transduces signals for nTreg development. We think that the controversy could come from the antigen specificity of nTregs. We hypothesize that TRA-specific Tregs and ubiquitous antigen-specific Tregs are generated in different locations. While the former relies more on thymic medulla for the induction of differentiation, the latter could take place in the cortex. Supporting this view, nTregs can develop in the thymus with major histocompatibility complex class II expressed only in the thymic cortical epithelium.[36] Since the *Relb*$^{-/-}$ thymus (lack of medulla) shows about 50% reduction of nTregs (our unpublished observation), we are currently investigating whether the remaining nTregs actually enrich ubiquitous antigen-specific TCRs. If this is true, it would further support our hypothesis that nTreg induction could take place in both the cortex and medulla, depending on their antigen specificity. This model would also allow us to study whether further nTreg education in the medulla is required for fully functional Treg maturation.

Thymocyte migration and T–mTEC interaction. Developing thymocytes traffic in the thymus in a well-organized process. Single-positive (SP) thymocytes after positive selection have to migrate into medulla to undergo negative selection and further maturation, given the critical role of mTECs in presenting TRAs. Chemotaxis directed by CCR7 and its ligands (CCR7L) has been shown to be important for cortex-to-medulla thymocyte migration.[37,38] Consistent with this, cortical DP thymocytes show increased CCR7 expression,[38,39] and CCR7 ligands (CCL21 and CCL19) are predominantly expressed in thymic medulla area, especially mTECs.[38,40] Defective cortex-to-medulla migration has been found in mice deficient for CCR7 or CCR7 ligands,[38] whereas the forced expression of CCR7 on premature thymocytes results in the relocation of DP thymocytes to the medulla.[37] Furthermore, impaired central tolerance was found by our group and others in CCR7- and CCR7L-deficient mice.[41,42]

Although the regulation of CCR7 expression on positively selected DP thymocytes remains largely unknown, our recent study indicates that the CCR7L expression on mTECs is partially dependent on the LTβR signaling pathway.[42] Actually, the LTβR-IKKα-alternative NF-κB signaling pathway has been well documented for the regulation of CCL21, CCL19 and other chemokines in secondary lymphoid organs and peripheral tissues.[43–45] We found that CCL21 and CCL19 are both significantly reduced in the thymi of *Ltbr*$^{-/-}$ mice as compared to wild-type thymi. Reanalysis of their expression in isolated mTECs

further demonstrates an intrinsic role of LTβR signaling in CCL21 and CCL19 thymic regulation. The reduction of CCL21 and CCL19 was further found to result in functional consequences—more SP $CD8^+$ thymocytes accumulated in the cortex in the $Ltbr^{-/-}$ mice as compared with wild-type mice.[42] This impaired medullary migration could, at least partially, contribute to the impaired negative selection of autoreactive thymocytes found in $Ltbr^{-/-}$ mice. It is worthwhile to note that while we found significant impairment of thymic negative selection in $Ltbr^{-/-}$ mice, the Mathis group found only a slight or no impairment of thymic negative selection.[46] This could be due to differences in the experimental models used. Whereas mice bearing a TCR transgene from ovalbumin-specific $CD8^+$ T cells (OT-I cells) were used in our study, mice carrying a transgene from ovalbumin-specific $CD4^+$ T cells (OT-II cells) were used by the Mathis group. It has been recently known that medullary migration of CD8 SP thymocytes relies more on CCR7 than on $CD4^+$ SP thymocytes.[47]

In addition to the cortex-to-medulla migration, in order to induce efficient negative selection, developing thymocytes and antigen-presenting cells have to form a relatively stable interaction,[48] a process might be influenced by both chemokines and adhesion molecules. We are using two-photon microscopy technique to identify the chemokines and adhesion molecules that are involved in the formation of stable T–mTEC interaction.

Canonical NF-κB pathway and autonomous thymocyte selection

Treg positive selection. While the alternative NF-κB pathway could influence nTreg development and function indirectly through the thymic medulla as discussed above, the classical NF-κB pathway has been shown to affect nTreg development autonomously.[49,50] The Zheng *et al.*'s study demonstrated reduced nTregs in peripheral $CD4^+$ T cells in $p50^{-/-}cRel^{-/-}$ mice, which could be influenced by both central and peripheral defects, whereas Schmidt-Suppria *et al.* clearly demonstrated a central defect of nTreg development in mice with IKKβ deficiency only in $CD4^+$ T cells. Interestingly, the small number of remaining Tregs in these mice contain many cells that have escaped Cre-mediated deletion of the loxP-flanked IKKβ alleles, which underscores the importance of IKKβ in the generation and/or maintenance of nTregs. The underlying mechanism remains unknown. However, it is not due to the inability of CD25 expression on T cells or reduced IL-2 production, as tested in the study.[49] Since nTregs are thought to be positively selected by agonist self-antigens in the thymus, which usually involves a strong activation of classical NF-κB (as discussed in more detail below), IKKβ could participate in TCR activation to regulate nTreg differentiation and/or maintenance. However, it is not known whether IKKβ is required quantitatively or qualitatively.

Thymocyte negative selection. Literature has indicated that NF-κB might play a pro-apoptotic role during thymocyte negative selection. In H-Y-specific TCR-transgenic male mice, DP thymocytes are largely absent as a result of negative selection. This is not due to impaired positive selection since this population is intact in female H-Y TCR-transgenic mice, in which no negative signaling to self-antigens is present. However, if NF-κB is partially inhibited by an IκB super-repressor, the DP thymocytes in the male mice are partially rescued.[51] This strongly supports a pro-apoptotic role for NF-κB in negative selection.

However, another study indicated that NF-κB could have an opposite role, and that an inhibition of NF-κB is required for negative selection.[52] The authors found that a novel IκB-like NF-κB inhibitor, IκBNS, is upregulated by peptides that trigger negative selection but not by those inducing positive selection (i.e., survival) or non-selecting peptides. IκBNS blocks transcription from NF-κB reporters. Retroviral transduction of IκBNS in fetal thymic organ culture enhances TCR-triggered cell death consistent with its function in selection. A more physiological model is required to confirm the role of IκBNS in thymocyte negative selection. Even so, it has been suggested that NF-κB could have both a pro-apoptotic and an anti-apoptotic role in thymocyte negative selection, which may depend on the timing and context.[53]

It must be noted that most studies about NF-κB and thymocytes selection were performed using the H-Y TCR or a superantigen-specific TCR as a model system. Therefore, whether and how NF-κB pathways autonomously participate in the establishment of central tolerance toward TRAs remains to be determined. TRAs-specific TCR-transgenic models will help to address the role of NF-κB in organ-specific central tolerance.

CONCLUSIONS AND PERSPECTIVES

Versatility and redundancy of NF-κB, to a large degree, complicate the study and hamper the progress in this field of research. Not only could NF-κB have multifaceted functions in one cell, but it could also participate in a wide variety of biological processes. Some NF-κB molecules are even critical for viability (e.g., embryo or perinatal lethality of $Rela^{-/-}$, $Ikkb^{-/-}$ and $Ikka^{-/-}$ mice, increased mortality in $Relb^{-/-}$ mice). Conditional targeting of specific NF-κB-related molecules at specific timing and in specific cell populations would help to resolve these problems.

Although much has been discovered about the role of NF-κB in T-cell central tolerance, how it actually works in detail remains largely unknown. For example, how is Aire expression regulated by NF-κB? Do other factors regulate Aire expression independently or together with NF-κB? Precisely how do NF-κB pathways control mTECs development, homeostasis and organization for both autoreactive T-cell negative selection and nTreg positive selection? Does NF-κB intrinsically control negative selection or positive selection of TRAs-specific autoreactive T cells or nTregs, respectively, and if yes, how do they control? Further works are required to reveal these mysteries.

The NF-κB family contains 15 hetero- or homodimer combinations that serve both redundant and specific functions. In addition, some IκB factors further expand the active transcriptional complex as direct nuclear mediators of NF-κB activity.[54] Given such a complex system, it is difficult to use conventional experimental approaches to address the functional differences and specificities among different NF-κB complexes. Therefore, various branches of biology such as systems biology, computational analysis, structural and molecular kinetics will be required to help address these complex questions.

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