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## NF- $\kappa$ B: Roles and Regulation In Different CD4<sup>+</sup> T cell subsets

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### Summary

The nuclear factor- $\kappa$ B (NF- $\kappa$ B) family of transcription factors plays important roles in various biological processes including apoptosis, stress response, immunity, and inflammation. NF- $\kappa$ B signaling is involved in both immune cell development and function, and it is critical in modulation of the immune response through the transcriptional regulation of cytokine and chemokine expression. An area of great interest in T-cell-mediated adaptive immunity is the ability of naive CD4<sup>+</sup> T cells generated in the thymus to differentiate into various subsets including T-helper 1 (Th1), Th2, Th17, Th9, follicular helper T (Tfh), Th22 and regulatory T (Treg) cells, upon encountering different pathogens and microenvironments. In this review, we discuss the role of NF- $\kappa$ B pathway in the development and functional divergence of the different helper T-cell subsets as well as in regulatory T cells.

### Keywords

NF- $\kappa$ B; T-cell subsets; T-cell development; T-cell signaling

### Introduction

T cells are critical elements of the vertebrate adaptive immune responses, where they function as both regulators and effectors of the immune response. Naive conventional T (Tconv) cells develop in the thymus and migrate to the periphery, where they encounter antigen or particular environmental conditions leading to the differentiation into effector subsets (Fig. 1). Notably, the NF- $\kappa$ B family of transcription factors has been shown to regulate various aspects of T-cell development, activation, differentiation, and survival.

The NF- $\kappa$ B family is comprised of five subunits: RelA (p65), RelB, c-Rel, NF- $\kappa$ B1 (p105), and NF- $\kappa$ B2 (p100) (1) (Fig. 2). The different subunits share an N-terminal Rel-homology domain (RHD), which is responsible for DNA-binding and homo- or hetero-dimerization. The p65, c-Rel, and RelB subunits contain a transcription activation domain and are hence capable of driving transcription. Under resting conditions, NF- $\kappa$ B dimers are sequestered in the cytoplasm by the inhibitory I $\kappa$ B proteins. Upon stimulation, the I $\kappa$ B kinase (IKK), which is comprised of IKK $\alpha$ , IKK $\beta$ , and the regulatory subunit NEMO, phosphorylates the I $\kappa$ B proteins, leading to their subsequent ubiquitination and degradation. As a result, NF- $\kappa$ B dimers are released from I $\kappa$ B inhibition and translocate to the nucleus where they bind to  $\kappa$ B-binding sites in the promoter or enhancer regions of target genes. In general, NF- $\kappa$ B activation occurs through two major pathways: the canonical and noncanonical pathways (Fig. 3). In the canonical pathway, IKK $\beta$  phosphorylation of I $\kappa$ B $\alpha$  leads to its degradation and the subsequent liberation of the NF- $\kappa$ B dimers. p65:p50 heterodimers are the major

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targets of the canonical pathway, but other combinations of dimers, including c-Rel containing dimers, can be involved in the pathway. In the noncanonical pathway, the NF- $\kappa$ B-inducing kinase (2) directly phosphorylates and activates IKK $\alpha$ . Activated IKK $\alpha$  phosphorylates p100, which results in proteosomal processing of p100 to p52. p52 forms a heterodimer with RelB and translocates to the nucleus to bind to  $\kappa$ B-binding sites. Activation of the NF- $\kappa$ B pathway regulates expression of a plethora of immunomodulatory factors, including cytokines, chemokines, adhesion molecules, antimicrobial factors, cell cycle regulators, and cell survival factors.

Stimulation of T cells through engagement of the T-cell receptor (TCR) and the co-receptor CD28 induces expression of activation-associated cell surface molecules, such as the adhesion molecule leukocyte function-associated antigen-1 (LFA-1) (3), as well as cytokines important for T-cell proliferation and survival, such as interleukin-2 (IL-2) (4). TCR, and CD28 activation of the NF- $\kappa$ B pathway is thought to contribute to this transcriptional program. Initial activation is mediated by p65-containing NF- $\kappa$ B complexes and is followed by delayed and sustained activation of the pathway through c-Rel-containing complexes (4). Various genetic studies, employing inhibition or deficiency of specific NF- $\kappa$ B subunits in T cells, have implicated NF- $\kappa$ B activity in the protection from apoptosis and the production of IL-2 (1). Furthermore, genetic models in mice have demonstrated an essential role of NF- $\kappa$ B in T-cell activation, expansion, and effector function during infection (1).

Activation of naive T cells also results in the rapid proliferation and simultaneous differentiation into effector subsets. T lymphocytes are classified into subsets based on TCR ( $\alpha\beta$  or  $\gamma\delta$ ) and coreceptor expression (CD4<sup>+</sup> or CD8<sup>+</sup>), cytokine profile, and divergent functions during an immune response. For the purposes of this review, we focus our discussion on the development and function of the various CD4<sup>+</sup> T-cell subsets.

CD4<sup>+</sup> T cells recognize peptide-major histocompatibility complex (MHC) class II complexes presented by antigen-presenting cells (APCs) and differentiate into different effector T-cell subtypes or regulatory T cells upon activation. Among CD4<sup>+</sup> T-helper cells, Th1 cells are responsible for immunity against intracellular pathogens primarily through secretion of interferon- $\gamma$  (IFN- $\gamma$ ), whereas Th2 cells promote the humoral immune response against extracellular pathogens through secretion of interleukin-4 (IL-4) and other cytokines. Th17, a recently discovered CD4<sup>+</sup> T-cell subtype, produces proinflammatory cytokines such as IL-17A, IL-17F, and IL-22, and mediates the immune response against extracellular bacteria. In some cases, particular T-cell subsets have also been shown to exacerbate disease conditions, e.g. Th17 cells contribute to autoimmune diseases such as multiple sclerosis. Another unique T-cell subset that has attracted tremendous interest in recent years is the regulatory T (Treg) cells, which are responsible for suppressing the immune response induced by effector T cells. Treg cells either develop directly in the thymus or differentiate from CD4<sup>+</sup> naive T cells in the periphery in response to specific environmental cues (inducible Treg cells). Finally, Th9, follicular helper T (Tfh) cells, and Th22 represent the most recently discovered CD4<sup>+</sup> subsets, which are actively being characterized at present.

Development of the different T-cell subsets is dependent on several known transcription factors: T-bet for Th1, GATA3 for Th2, forkhead box protein 3 (FoxP3) for Treg, and ROR $\gamma$ t and ROR $\alpha$  for Th17 cells. However, it is also becoming apparent that combinatorial activation of distinct signaling pathways and the concomitant mobilization of multiple transcription factors contributes to lineage determination. As previously mentioned, transcription factors of the NF- $\kappa$ B family are also involved in various aspects of T-cell development and function, and recent work has helped further define the unique functions of this pathway in the differentiation and functional divergence of the various T-cell subsets (5). In this review, we provide a comprehensive discussion of the current literature on the

role and regulation of NF- $\kappa$ B in different T-cell subsets and speculate on the impact of NF- $\kappa$ B regulation in the overall immune response.

## Differentiation of Th1 and T2 cells

CD4<sup>+</sup> T cells produced in the thymus migrate to the periphery where they encounter antigens. Upon stimulation, naive CD4<sup>+</sup> T cells differentiate into distinct subsets of effector cells, which allow for tailored immune responses against specific antigens. Th1 and Th2 cells are two major CD4<sup>+</sup> effector T-cell subsets. As discussed earlier, the major role of Th1 cells is in immune response against intracellular viral or bacterial pathogens, predominantly through the production of IFN- $\gamma$ . Th2 cells are involved in the response to extracellular pathogens, but they have also been implicated in allergic reactions. Th2 cells are characterized by their production of IL-4, as well as IL-5, IL-6, IL-9, IL-13, and IL-25.

Th1 cell differentiation is initiated by TCR engagement with a peptide-MHC class II complex presented by DCs or macrophages in response to infection with an intracellular pathogen. This TCR stimulation induces low-level expression of the transcription factors T-bet and GATA3. T-bet increases the surface expression of the IL-12 receptor, thus enhancing the sensitivity of cells to IL-12 and promoting further T-cell differentiation through IL-12 and IFN- $\gamma$  produced by APCs. IL-12 stimulation of T cells activates the transcription factor signal transducer and activator of transcription 4 (STAT4). In addition, receptor engagement of IFN- $\gamma$  induces activation of STAT1, which subsequently further increases the level of T-bet. Finally, cooperation of T-bet, STAT4 and STAT1 induces high level of IFN- $\gamma$  production by the T cell (Th1). In addition to the previously described network of transcription factors, several studies have described a role of NF- $\kappa$ B in Th1 differentiation. In transgenic mice expressing a non-degradable form of I $\kappa$ B specifically in T cells, Th1 responses were significantly impaired due to diminished NF- $\kappa$ B activation (6). Also, c-Rel-deficient mice showed defective Th1-mediated immune responses and abrogated IFN- $\gamma$  production by T cells (7). However, T-bet expression in those T cells was intact, implying that c-Rel might be downstream of T-bet and consequently be important for T-bet-induced Th1 differentiation. On the other hand, RelB-deficient T cells exhibited a defect in Th1 differentiation, but this was due to decreased expression of T-bet (8). RelB is thought to mediate STAT4 upregulation, which in turn, is responsible for IFN- $\gamma$  induction of T-bet.

RelA (p65) is recruited to highly conserved non-coding sequences (CNSs) in the enhancer region in the IFN- $\gamma$  locus and in the absence of RelA, IFN- $\gamma$  production in Th1 cells is greatly impaired (9). Recruitment of RelA to the enhancer region is dependent on T-bet expression in Th1 cells. STAT4 is co-recruited to the CNS sites suggesting that RelA and STAT4 may cooperate to remodel the IFN- $\gamma$  locus and induce the expression of IFN- $\gamma$ . While these findings establish a critical role for RelA in Th1 differentiation and function, it still remains unknown whether RelA binds to the CNS regions as a heterodimer with p50 or c-Rel, or as a homodimer. According to chromatin immunoprecipitation (ChIP) analysis, p50 binding seems to be limited to only one CNS site suggesting that the other site might bind to a p65:c-Rel complex. Interestingly, p50-deficient CD4<sup>+</sup> T cells are able to express T-bet and produce IFN- $\gamma$  in Th1 differentiation, implying that RelA might work either as a homodimer or a heterodimer with c-Rel in the IFN- $\gamma$  locus (10). Taken together, these studies establish an essential role for NF- $\kappa$ B members in the development and function of Th1 cells, especially in the production of IFN $\gamma$ .

Th2 development is initiated by IL-4 stimulation, which leads to activation of the transcription factor STAT6. Furthermore, IL-4 signaling enhances the expression of the *IL-4* gene via activation of NFAT, AP-1 and NF- $\kappa$ B, and induces expression of GATA3, a master

regulator of Th2 differentiation. GATA3 governs the expression of another transcription factor, c-Maf, which also helps the induction of IL-4. However, overexpression of c-Maf alone in naive CD4<sup>+</sup> T cells did not induce Th2 differentiation in Th1-skewing conditions (11) unlike the situation seen upon overexpression of GATA3 (12). Therefore, STAT6, GATA3, and c-Maf work cooperatively to induce a Th2 program and stabilize the features of Th2 cells. Importantly, NF- $\kappa$ B has been reported to be essential for the expression of the *GATA3* gene (10). CD4<sup>+</sup> T cells from p50-deficient mice were unable to express GATA3 under Th2-differentiating conditions *in vivo* and *in vitro*. Consequently, impaired Th2 development in p50-deficient mice led to a defective allergic airway inflammatory response. These findings demonstrate a crucial role for p50 in Th2 differentiation through the transcriptional regulation of GATA3 expression.

Expression of the *IL-4* gene, which is crucial for Th2 differentiation, is known to be regulated by two mechanisms. First, GATA3-induced chromatin remodeling opens up the gene locus of *IL-4* in the process of Th2 differentiation. Second, IL-4 is induced by TCR engagement in mature Th2 cells. NF- $\kappa$ B has been shown to bind two major enhancer sites in the *IL-4* locus and it participates in induction of IL-4 in cooperation with nuclear factor of activated T cells (NFAT) (13). Since NF- $\kappa$ B is also involved in induction of GATA-3, it remains unclear whether the role of NF- $\kappa$ B in Th2 differentiation is a direct result of TCR-induced NF- $\kappa$ B activating *IL-4* gene expression or if it acts primarily through its effect on GATA3 expression.

## Development of natural regulatory T (nTreg) cells in the thymus

The immune system is a carefully balanced system with tight regulation of immune cell function. When the balance is disrupted, it can lead to disease. Since the original discovery of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells by Sakaguchi and colleagues (14), there has been much progress in understanding the role of Treg cells in immune homeostasis and function. The discovery of the Treg-specific transcription factor FoxP3 has accelerated this progress. FoxP3<sup>+</sup> Treg cells are essential for suppressing excessive immune response against self or non-self-antigens. Human immunodysregulation, polyendocrinopathy, and enteropathy X-linked (IPEX) syndrome patients have mutations in the FoxP3 locus, resulting in a lack of functional FoxP3<sup>+</sup> Treg cells. The symptoms of IPEX syndrome resemble those of Scurfy mice, which have a loss-of-function mutation in the FoxP3 gene. Scurfy mice have systemic lymphoproliferative disease in multiple organs and die within 2–3 weeks (15). IPEX patients suffer from various symptoms such as autoimmune endocrinopathies, eczema, diabetes, and severe enlargement of the secondary lymphoid organs (16).

In addition to playing a protective role against autoimmunity, Treg cells help maintain immune homeostasis in various settings including in response to commensal microbiota or food allergens at mucosal surfaces. Treg cells also suppress experimental graft-versus-host disease (GvHD) in mice (17), leading to the exploration of Treg modulation as a potential therapeutic targeting strategy. On the other hand, altered Treg function in some cancers facilitates immune evasion, thereby promoting the exploration of alternative approaches towards cancer immunotherapies (18). A recent publication from Rudensky and colleagues (19) reported a role of Treg cells in maternal-fetal immune tolerance and shed light on the contribution of Treg cells in situations such as pre-eclampsia during pregnancy. Since Treg cells are important in many immune disorders, it has been a major interest to study how Treg cells differentiate and function.

Treg cells are classified into two major classes based on their developmental origin. Treg cells can be generated in the thymus through the natural selection process and these cells are referred to as natural Treg (nTreg) or thymic Treg (tTreg) cells. Alternatively, conventional

naive T cells can differentiate into FoxP3<sup>+</sup> Treg cells in the periphery upon exposure to non-self-antigens such as commensal microbiota, food, or allergens. These Treg cells generated in the periphery are generally referred to as inducible Treg (iTreg) cells. However in this review, we primarily focus on the thymic development of nTreg cells.

T-cell progenitors are educated by exposure to various self-antigens presented by APCs in the thymus during positive selection. Only T cells that successfully detect MHCs bound to moderate-affinity peptides will receive a survival signal. However, T cells carrying high-affinity T-cell receptors (TCRs) against self-antigens are eliminated by apoptosis through negative selection. T cells escaping negative selection are released from the thymus and migrate to the periphery. However, a small number of self-reactive T cells with intermediate affinity for self-antigens can escape the elimination process during negative selection and differentiate into FoxP3<sup>+</sup> Treg cells.

The mechanism of how Treg cells are selected in the thymus has been extensively studied, but several questions still remain to be answered. A two-step model has been suggested for thymic Treg development with the first step being TCR-dependent, while the second step is TCR-independent (20). TCR signaling in thymocytes triggered by the TCR-MHC class II interaction induces downstream signaling pathways that elicit NF- $\kappa$ B, NF-AT, and mammalian target of rapamycin (mTOR)/AKT activity. It has been suggested that TCR signaling in Treg development is different from the TCR signaling that occurs during the development of Tconv cells. Deficiency of several mediators of TCR signaling such as TAK1, BCL10, CARMA1, PKC $\theta$ , and IKK $\beta$  significantly reduced or abrogated the number of Treg cells generated in the thymus without affecting the generation of Tconv cells (21–25). These molecules are known to be important for signal transduction upstream of NF- $\kappa$ B activation, which suggests a possible role for NF- $\kappa$ B in thymic Treg development. Interestingly, thymic Treg generation in TAK1- or CARMA1-deficient mice can be rescued by crossing these strains to transgenic mice expressing a constitutively active mutant form of IKK $\beta$  under the control of a proximal Lck promoter (IKK $\beta$ -Tg), demonstrating that the defective Treg development is due to impaired NF- $\kappa$ B activation in these knockout mice (26). Also, overexpression of a constitutively active form of IKK $\beta$  generated thymic FoxP3<sup>+</sup> Treg cells in RAG-deficient TCR-transgenic mice (OT-II and P14-TCR), which normally do not produce nTreg cells in the thymus. This demonstrates that forced activation of NF- $\kappa$ B can bypass the requirement of self-antigen recognition via TCR engagement. Together, these findings strongly suggest that NF- $\kappa$ B activation is critical for thymic Treg development.

The mechanism of how the FoxP3 locus becomes more accessible to the transcription machinery during thymic selection has been a question of major interest. Enhancer regions in FoxP3 locus have been suggested to have an important role in inducing FoxP3 transcription. Three highly conserved non-coding sequences have been identified in the FoxP3 locus (32, 33): CNS1, CNS2, and CNS3. CNS1 contains a TGF- $\beta$ -responsive element and binding sites for AP1, NFAT, and SMAD3, and it is known to be important for iTreg differentiation but not for thymic development of nTreg cells (34). CNS2 has binding sites for several transcription factors including CREB, CBF $\beta$ , and STAT5 and is necessary for maintenance of FoxP3 expression (34). The NF- $\kappa$ B subunit c-Rel binds to CNS3 and plays a major role in thymic and peripheral FoxP3 expression and Treg differentiation (26, 34). The CNS2 and CNS3 enhancer sequences have been reported to contain highly conserved CpG-rich regions which are fully demethylated in nTreg cells but highly methylated in Tconv cells as well as iTreg cells (32, 34). Demethylation in these CNS sequences is believed to be necessary for stable expression of FoxP3. It has been proposed that c-Rel binds to the CNS3 region as a homodimer, but it is not yet clear if c-Rel synergizes with other transcription factors at the FoxP3 locus. It will be exciting to further

explore the interplay between transcription factor activation and the modification of chromosome structure that leads to FoxP3 expression and Treg differentiation.

## NF- $\kappa$ B in Treg suppression of the immune response

TCR stimulation is required for thymic Treg development and is also necessary for Treg function, but the signaling pathways governing the function of Treg cells are quite complex. PDK1 is an important protein kinase that integrates TCR stimulation and CD28 costimulation and induces activation of PKC $\theta$ , followed by the formation of CARMA1/MALT1/BCL10 complex, and subsequently NF- $\kappa$ B activation (47) (Fig. 4). PDK1 was conditionally ablated in CD4<sup>+</sup> T cells using the Cre-Lox system in mice to study the role of PDK1 in CD4<sup>+</sup> T cells. Interestingly, these mice spontaneously developed colitis, even though activation of effector CD4<sup>+</sup> T cells was defective due to the absence of PDK1. It was shown that colitis was due to dramatically increased numbers of  $\gamma\delta$ T cells in the colon, and crossing the PDK1-deficient mice with a TCR $\delta$ -knock-out mouse completely abolished the colitis (48). In line with these findings, PDK1-deficient Treg cells did not have suppressive activity both *in vitro* and *in vivo* in a transfer colitis model. As wildtype Treg cells are able to inhibit proliferation of  $\gamma\delta$ T cells, the increased number of  $\gamma\delta$ T cells in the PDK1-deficient mice was due to the lack of functional Treg cells. These studies suggested that PDK1 likely has an important role in Treg function. As the lack of PDK1 prevents NF- $\kappa$ B activation, it is possible that NF- $\kappa$ B might be important in the suppressive function of Treg cells. However, because PDK1 activation can induce other downstream signaling pathways such as mTOR/Akt (Fig. 4), it is possible that these other pathways are important in Treg function.

A recent paper from Sun and colleagues (49) showed that the Ubc13-IKK axis has a positive role in Treg suppressive function. Ubc13 is an E2 ubiquitin-conjugating enzyme that specifically conjugates Lys63-linked polyubiquitin chains, which are known to have a role in activation of IKK and NF- $\kappa$ B (50). Conditional ablation of Ubc13 in FoxP3<sup>+</sup> Treg cells led to multi-organ inflammation due to uncontrolled activation of Tconv cells (49). Ubc13-deficient Treg cells could not suppress inflammatory colitis induced by adoptive transfer of naïve Tconv cells into lymphopenic recipients, demonstrating that Ubc13 depletion abrogates the suppressive function of Treg cells *in vivo*. When Ubc13 Treg-knockout mice were crossed to transgenic mice expressing a constitutively active form of IKK $\beta$  under the control of a *loxP*-flanked stop cassette, the suppressive function of Ubc13-deficient Treg cells was rescued, as indicated by a decrease in the frequency of memory and effector-like T cells in lymphoid organs (49). These results, coupled with the findings on PDK1-deficient mice (48), suggest that NF- $\kappa$ B might be a key regulator determining Treg function.

In contrast to the previous findings, Dustin and colleagues (51) reported that PKC $\theta$ , a downstream target of PDK1, has a negative role in Treg function. This is a surprising finding as PKC $\theta$  is activated upon TCR engagement and is believed to be critical for activation of NF- $\kappa$ B, NFAT, and mTOR/Akt pathways. Consistent with the fact that TCR signaling and NF- $\kappa$ B activation are required for nTreg differentiation, PKC $\theta$ -deficient mice showed reduced numbers of Treg cells. However, surprisingly, inhibition of PKC $\theta$  using a chemical inhibitor increased the suppressive capacity of Treg cells suggesting that PKC $\theta$  exerts a negative role in the suppressive function of Treg cells (22, 24). A possible explanation might be the different experimental approaches used in the two studies. Whereas PKC $\theta$  was blocked transiently using a chemical inhibitor, PDK1 was removed permanently using genetic ablation. However, further studies will be required to resolve the role of PKC $\theta$  in Treg cells.

## NF- $\kappa$ B in the development of Th17 Cells

As mentioned previously, Th17 cells are a CD4<sup>+</sup> T-cell subset that produces proinflammatory cytokines such as IL-17A, IL-17F, and IL-22, and mediates the immune response against extracellular bacteria. Recently, a possible role of NF- $\kappa$ B in the regulation of ROR $\gamma$ t gene expression in the differentiation and function of Th17 cells was demonstrated (52). Earlier studies showed that c-Rel-deficient mice were resistant to experimental autoimmune encephalomyelitis (EAE), a mouse model for multiple sclerosis, which is mediated by T-helper cell subsets including Th17 cells (7). This finding suggested a possible role of c-Rel in the inflammatory Th17 response, but as c-Rel is involved in the development and function of various immune cells, the possible role of other cell types cannot be discounted. More recently, c-Rel- or p65-deficient T cells showed defective IL-17 gene expression and Th17 cell differentiation (52). In addition, gene expression of ROR $\gamma$ t and ROR $\gamma$ , an isoform of ROR $\gamma$ t, was significantly reduced in c-Rel-deficient T cells. ROR $\gamma$ t and ROR $\gamma$  are encoded by a single gene, *Rorg* and share the last 9 exons. However, their first 100 nucleotides are different, which leads to different N-terminal sequences after translation. ROR $\gamma$ t and ROR $\gamma$  share the same DNA-binding domain and ligand-binding domain. Like ROR $\gamma$ t, ROR $\gamma$  was shown to bind to the *Il17a* promoter region, suggesting that ROR $\gamma$  might also be involved in Th17 differentiation. Gene expression of ROR $\gamma$ t and ROR $\gamma$  are controlled by two different promoters, both of which were found to contain NF- $\kappa$ B-binding sites. A series of deletion and mutation experiments showed that c-Rel binds to these sites and that this binding was required for the expression of ROR $\gamma$ t and ROR $\gamma$ . This was further confirmed by chromatin immunoprecipitation analysis in Th17 cells. p65 also co-localized with c-Rel at the same binding sites. These results indicate that c-Rel, possibly together with p65, activates the gene expression of ROR $\gamma$ t and ROR $\gamma$ . However, evidence for c-Rel directly controlling *IL-17a* gene expression by binding to its promoter has not yet been provided. It has been hypothesized that the c-Rel:p65 dimers move into the nucleus and bind to the promoters of ROR $\gamma$ t and ROR $\gamma$  as a complex with other transcription factors such as NFAT and STAT. A similar hypothesis has been put forward regarding the control of the FoxP3 promoter in Treg cells, where each transcription factor participates in various signaling pathways but together can have a unique role in a distinct pathway as a complex with multiple transcription factors, called an 'enhanceosome' (53).

In contrast to the previously mentioned findings, Visekruna *et al.* (54) reported that c-Rel is not required for Th17 cell differentiation, a discrepancy that has been ascribed to the absence of anti-IL-2 blocking antibody in their Th17 *in vitro* differentiation condition. NF- $\kappa$ B is known to be required for IL-2 production, so c-Rel-deficient T cells may produce less IL-2 than wildtype T cells. Since IL-2 is inhibitory for Th17 cell induction, the presence of IL-2 in the wildtype T cell cultures may have masked the difference between wildtype and c-Rel-deficient T cells in the *in vitro* Th17 differentiation assay in the study by Visekruna *et al.* (54).

Powolny-Budnicka *et al.* (55) reported a role of RelA (p65) and RelB in lymphotoxin (LT)-dependent IL-17 production in  $\gamma\delta$ T cells. While they focused on  $\gamma\delta$ T cells in this study, they also examined Th17 cells. They showed that differentiation of IL-17-producing  $\gamma\delta$ T cells in the thymus is dependent on the LT $\beta$  receptor (LT $\beta$ R), RelA, and RelB. RelA controls expression of LT ligands in accessory thymocytes to regulate IL-17 production in  $\gamma\delta$ T cells. On the other hand, RelB may act downstream of LT $\beta$ R and increase the mRNA level of ROR $\gamma$ t, resulting in the differentiation of IL-17-producing  $\gamma\delta$ T cells. However, they claimed that differentiation of Th17 cells is not dependent on either LT $\beta$ R or activation of RelA or RelB. CD4<sup>+</sup> T cells deficient in RelA or RelB did not show increased mRNA expression of ROR $\gamma$ t in their *in vitro* Th17 differentiation assay. Chen and colleagues (52), on the other hand, showed that while the noncanonical NF- $\kappa$ B pathway including RelB is

not involved in ROR $\gamma$ t expression in Th17 differentiation, c-Rel and p65 (RelA) directly bind to the ROR $\gamma$ t promoter region and upregulate its mRNA expression. Discrepancies between these two group's studies could be explained by the experimental conditions of the *in vitro* Th17 differentiation assay. The *in vitro* differentiation cultures used in Powolny-Budnicka *et al.*'s study (55) were performed in the absence of anti-IL-2. As p65 (RelA) is known to be important in IL-2 production (56), the p65-deficient T cells may produce less IL-2 than wildtype T cells. Differences in Th17 differentiation might have been masked by the effect of inhibitory IL-2 in the culture of the wildtype cells. Ultimately, the role of the different NF- $\kappa$ B subunits in differentiation of Th17 and  $\gamma$  $\delta$ T cells is still not clear and needs to be further studied. Furthermore, the interplay between NF- $\kappa$ B and other transcription factors in controlling the differential expression of ROR $\gamma$ t in T-cell subsets remains an area of great interest.

## NF- $\kappa$ B in Th9 development

Th9 is a subset of T-helper cells producing IL-9, but their development and function remain poorly understood. A recent report suggested a mechanism of Th9 induction through OX40 signaling (57). OX-40 is a T-cell costimulatory molecule that belongs to the TNF-receptor family. Ligation of OX40 strongly induced Th9 differentiation in the presence of IL-4 and TGF- $\beta$  and activated both canonical and noncanonical NF- $\kappa$ B pathways. However, Th9 induction was not affected when the canonical pathway was repressed by using transgenic mice overexpressing a nondegradable form of I $\kappa$ B or a chemical inhibitor of IKK activity (BAY 11-7082). On the other hand, p52-deficient T cells could not differentiate into Th9 cells. Also, when p52-RelB was overexpressed in wildtype CD4<sup>+</sup> T cells by retrovirus-mediated gene transfer, cells were able to convert into Th9 cells even without OX40 ligation in the presence of IL-4 and TGF- $\beta$ . OX40 signaling activates the ubiquitin ligase TRAF6, which activates NF- $\kappa$ B-inducing kinase (2). This results in the processing of p100 to generate p52, which then forms a heterodimer with RelB. These results suggest that the noncanonical NF- $\kappa$ B pathway via the RelB:p52 heterodimer might be important in Th9 differentiation. Finally, it has been suggested that IL-9 expression in T cells is regulated by NF- $\kappa$ B, thereby implicating the pathway in the function of these cells (58). While many questions concerning the Th9 subset of CD4<sup>+</sup> T cells remain unanswered, these findings represent the first steps towards a better understanding of the development and function of Th9 cells.

## Follicular helper T cells (Tfh)

In the germinal center (GC), B cells exposed to antigens differentiate and mature to produce antibodies against specific antigens. Another T-cell subset called follicular helper T cells (Tfh) is critical for the maturation of germinal center B cells. Tfh cells express the chemokine receptor CXCR5, which enables Tfh cells to migrate to B-cell follicles. Multiple signals from Tfh cells are needed to enable full differentiation of B cells. IL-21 produced by Tfh cells and CD40L, and programmed death-1 (PD1) expressed on the surface of Tfh cells, are important for B-cell differentiation. Also, Tfh cells express high level of ICOS, and the resulting costimulatory signal through the interaction between T-cell ICOS and B-cell ICOSL is critical for B-cell maturation (59).

For Tfh development, T cells first need to be initially activated by DCs in the T-cell zone of the follicle. Then, these Tfh cell precursors interact with B cells at the border of T-B cell zones. In addition to TCR/CD28 signals, ICOS costimulation through B cells has been shown to be required for Tfh development (60–63). Furthermore, defective Tfh development was reported to occur in NIK-deficient mice (64), but it was not due to a T-cell intrinsic deficiency. NIK was required for maintaining the high-level expression of ICOSL in B cells,



which is essential for Tfh development. Noncanonical NF- $\kappa$ B members RelB and p52 have been shown to bind to a  $\kappa$ B-binding site in the ICOSL promoter region and shRNA-mediated knock-down of RelB in B cells impaired ICOSL induction in response to BAFF stimulation (64). These results suggest that the noncanonical NF- $\kappa$ B pathway might play an important role in Tfh development by regulating the expression of ICOSL in B cells.

In another report using NF- $\kappa$ B1-deficient OT-II mice, Tfh development was seen to be defective upon immunization with alum-precipitated OVA peptides (65). NF- $\kappa$ B1 deficiency particularly impaired CXCR5 expression. In addition, there were lower numbers of Tfh cells in these mice, which resulted in development of fewer germinal center B cells in response to OVA immunization. These findings suggest a role of NF- $\kappa$ B in Tfh development.

In addition to its role in stimulating B cells to differentiate, IL-21 has been shown to be important in the development of Tfh cells, and differentiated Tfh cells are dependent on IL-21 for their growth and survival (66, 67). IL-21 production was impaired in c-Rel-deficient mice, and the development of Tfh and germinal-center B cells was consequently inhibited in those mice (68). c-Rel was shown to bind to the IL-21 promoter, implying that c-Rel directly regulates the transcription of IL-21. Taken together, these findings highlight the role of NF- $\kappa$ B family members in regulating various steps in the development and function of Tfh cells.

## Perspectives

This review discusses the role of NF- $\kappa$ B in different CD4<sup>+</sup> T cell subsets, including Th1, Th2, Treg, Th17, Th9, and Tfh cells. NF- $\kappa$ B family members are critical transcription factors that regulate a plethora of genes in different biological processes. While the molecular details of NF- $\kappa$ B activation downstream of TCR/CD28 signaling and the role of the NF- $\kappa$ B pathway following TCR activation have been extensively studied, the differential functions of NF- $\kappa$ B during development and activation of the specific T-cell subset is a newer field of study. The utilization of the different NF- $\kappa$ B subunits and the synergy of the NF- $\kappa$ B pathway with other signaling pathways contribute to the complex transcriptional programs that allow for the differentiation and functional divergence of precursor cells into the various T-cell subsets. A major question that stems from the studies discussed in this review is how a common stimulus can direct different gene expression profiles downstream of NF- $\kappa$ B in response to different environmental conditions, thereby creating the unique effector profiles of the different T-cell subsets. Recently, a number of studies have suggested that a coordinated network of multiple transcription factors could regulate the expression of specific sets of genes in response to specific stimulation and environmental conditions. By studying the interplay between signaling pathways and the resultant networks formed by NF- $\kappa$ B and other transcription factors in response to various stimuli, we hope to better understand the tight regulatory mechanisms involved in the development and function of immune cells. Consequently, we may be able to better anticipate conditions that could contribute to dysregulation of immune cell function and immune responses, and perhaps use our understanding of lineage decisions in the design of therapeutics that specifically target the dysregulated cell types or cell functions.

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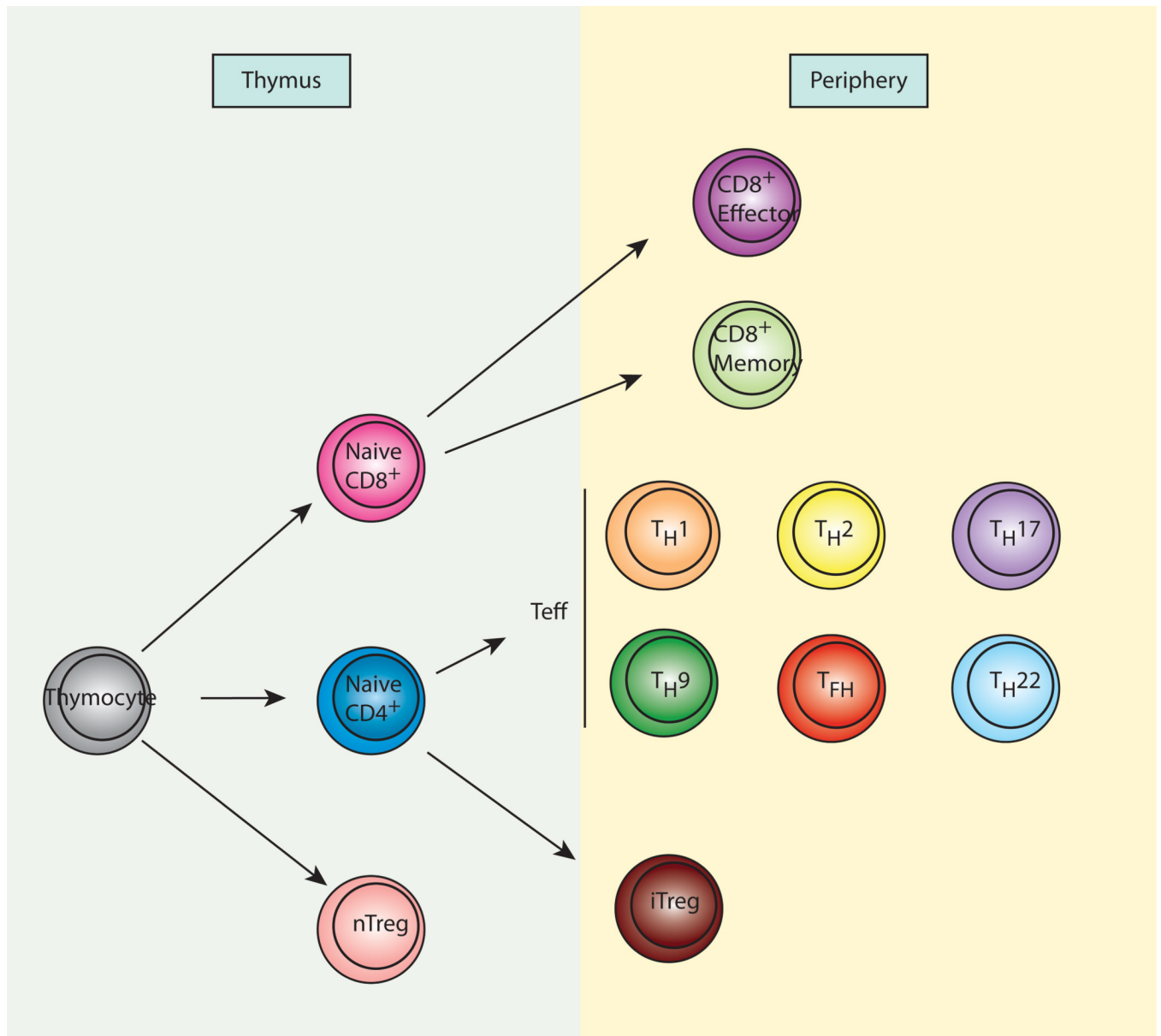
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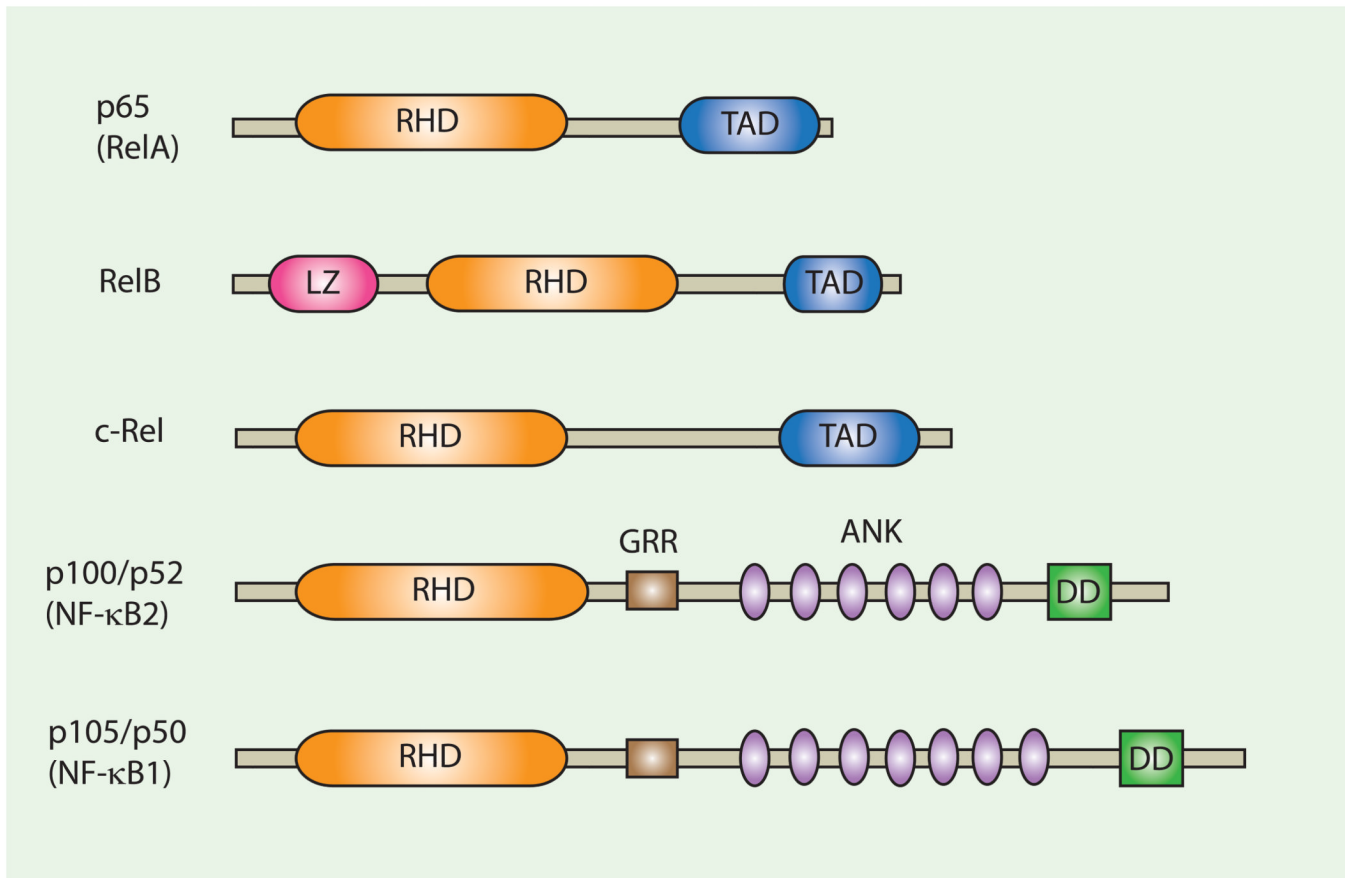
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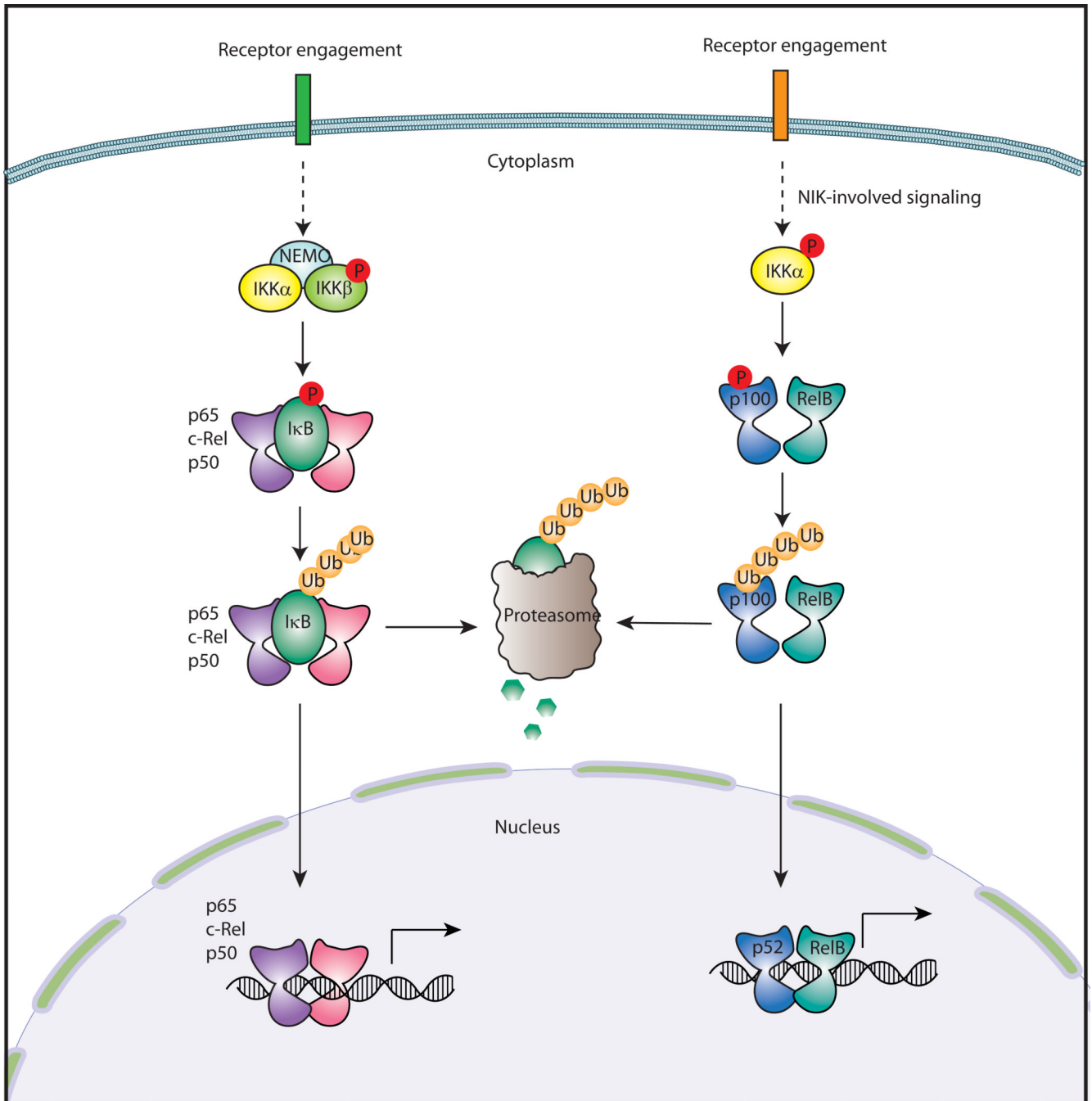
**Fig. 1. CD4<sup>+</sup> T-cell subsets**

In the thymus, naive CD4<sup>+</sup> and CD8<sup>+</sup> T cells develop from thymocytes. Naive CD4<sup>+</sup> T cells from the thymus migrate to the periphery, where they can differentiate to various subsets of effector cells upon encountering specific antigens. The subsets include Th1, Th2, Th17, Th9, Tfh, and Th22. Naive CD8<sup>+</sup> T cells also migrate to the periphery and differentiate to effector cells or memory cells upon encountering antigens. A small population of thymocytes differentiates into regulatory T cells (nTreg) in the thymus. In addition, some naive CD4<sup>+</sup> T cells differentiate into regulatory T cells (iTreg) in specific microenvironments.



**Fig. 2. Members of the NF-κB/Rel family**

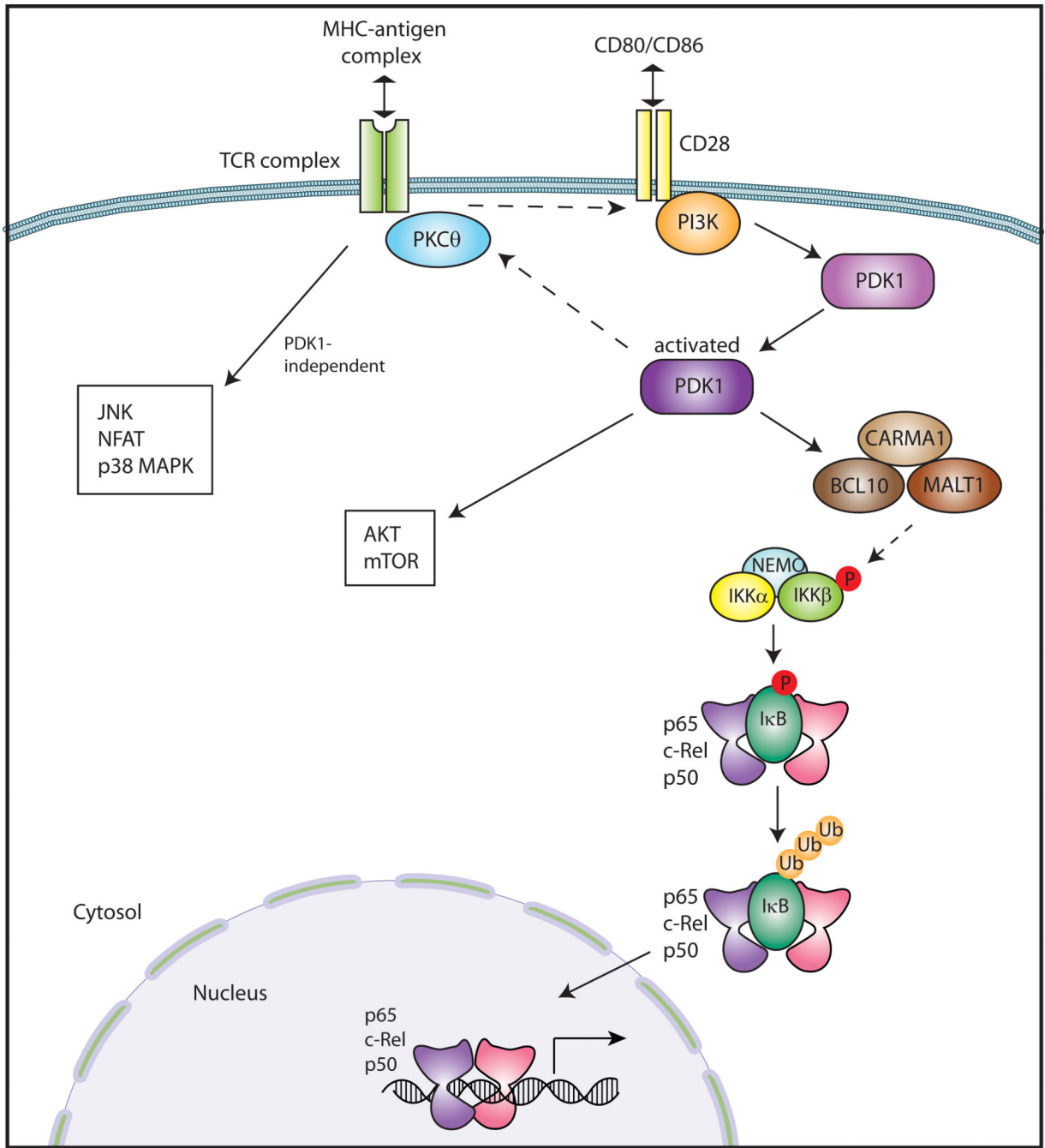
Schematic depiction of members of the NF-κB transcription factor family. RHD, Rel homology domain; TAD, transactivation domain; LZ, leucin zipper domain; GRR, glycine-rich region; DD, death domain; ANK, ankyrin repeats.



**Fig. 3. Canonical and noncanonical pathways of NF- $\kappa$ B activation**

NF- $\kappa$ B activation occurs through two different pathways: canonical and noncanonical. In the canonical pathway, the IKK complex, composed of IKK $\alpha$ , IKK $\beta$ , and the regulatory subunit NEMO, phosphorylates I $\kappa$ B, leading to its ubiquitination and proteasomal degradation. A NF- $\kappa$ B dimer, which is comprised of p50, p65, or c-Rel, is released from I $\kappa$ B and translocates into the nucleus, where it binds to a  $\kappa$ B-binding site for regulation of gene expression. In the noncanonical pathway, IKK $\alpha$  phosphorylates p100 resulting in its ubiquitination and partial processing into p52. A heterodimer of p52 and RelB translocates into the nucleus and binds to its binding sites. P, phosphorylation; Ub, ubiquitination.





**Fig. 4. TCR/CD28 signaling pathway**

A TCR complex on the surface of T cells recognizes a specific antigen-MHC complex on an antigen-presenting cell (APC). At the same time, a costimulatory receptor CD28 interacts with CD80 or CD86 on the APC. Upon TCR/CD28 stimulation, PI3K activates PDK1 leading to subsequent activation of PKCθ. This results in the formation of the complex consisting of CARMA1, BCL10, and MALT1, that activates the IKK complex. Phosphorylation of IκB by the IKK complex leads to its degradation, resulting in the liberation of NF-κB dimers. NF-κB dimers translocate into the nucleus and regulate the expression of target genes. PDK1 activates the Akt/mTOR pathway as well as NF-κB. TCR

stimulation also leads to activation of JNK, NFAT, and p38 MAPK in a PDK-independent manner.