Trends in Immunology

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Review

NF-κB and Extrinsic Cell Death Pathways – Entwined Do-or-Die Decisions for T cells

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NF-κB signaling is required at multiple stages of T cell development and function. The NF-κB pathway integrates signals from many receptors and involves diverse adapters and kinases. Recent advances demonstrate that kinases controlling NF-κB activation, such as the IKK complex, serve dual independent functions because they also control cell death checkpoints. Survival functions previously attributed to NF-κB are in fact mediated by these upstream kinases by novel mechanisms. This new understanding has led to a refined view of how NF-κB and cell death signaling are interlinked and how they regulate cell fate. We discuss how NF-κB activation and control of cell death signaling by common upstream triggers cooperate to regulate different aspects of T cell development and function.

Highlights

Kinases such as IKK serve dual functions in activating NF-κB and in repressing cell death pathways independently of NF-κB activation.

Protection of mature thymocytes from TNF-induced cell death appears to be exclusively mediated by direct repression of RIPK1 by the IKK complex, and not by NF-κB activation.

The development and homeostasis of conventional and regulatory T cells (Tregs) relies on signaling via distinct TNF receptor superfamily (TNFRSF) members that activate specific NF-κB dimers.

Naïve T cell survival is predominantly mediated by RelA and by TNFRSF members including TNFR1 and CD27.

Based on mouse studies, Tregs require c-Rel for development and RelA for functional competence. TCR, TNFR2, GITR, and OX40 receptors are key triggers of NF-κB activity in Tregs.

Cell death and NF-κB signaling pathways are under developmental regulation in T cells at the level of RIPK1 and MLKL expression; this determines how and when signals are delivered during T cell ontogeny, and what modes of cell death can be triggered.

NF-κB Signaling Pathways Regulating T Cell Homeostasis, Development, and Function

There has been considerable interest over the years in understanding the role of NF-κB signaling in the development, function, and homeostasis of vertebrate T cells. The pathways of NF-κB activation are highly complex. There are a multitude of surface receptors that can activate NF-κB through one of two distinct pathways, and activate transcription factor dimers from a family of five different monomers. The two pathways of NF-κB activation and how surface receptors connect to them are described in detail in Figure I in Box 1. Given this complexity, it is perhaps unsurprising to find that NF-κB mediates functions that vary between different T cell subsets, and between different stages of differentiation in the thymus and periphery. Recent studies reveal that different types of NF-κB dimers have specific roles in the development of naïve, activated, and regulatory (Treg) T cells. Furthermore, other studies show that the kinases responsible for directly triggering NF-κB activity in fact have additional substrates that are involved in controlling cell death checkpoints [1]. These recent advances have led to an understanding that not all NF-κB dimers are equal, and have identified a new and previously unappreciated function of death signaling in controlling T cell homeostasis and development – functions mistakenly attributed to NF-κB in the past. This review discusses how the parallel activities of NF-κB and death signaling control T cell development, tune the long-term survival of naïve T cells, guide the development and function of Tregs, and expose activated T cells to alternative modes of cell death.

NF-κB in Thymic Development – Guilty by Association but Redundant

The T cell receptor (TCR) has long been appreciated as a potent activator of NF-κB during T cell activation, and TCR signaling plays a central role in guiding different stages of T cell development in the thymus that involve selection of the TCR repertoire [2]. Previous studies have implied that NF-κB plays a role downstream of the **pre-TCR complex** (see Glossary) in $CD4^{-}CD8^{-}$ double-negative thymocytes [3], and downstream of the TCR for the selection of CD8 lineage cells from $CD4^{+}CD8^{+}$ double-positive (DP) thymocytes [4–6]. The most striking phenotype caused by interfering with NF-κB pathways occurs when upstream triggers, such as the **IKK** complex, **TAK1**, or **LUBAC** (Figure I in Box 2), are genetically ablated in *Cd4*^{cre}***Chuk***$^{fl/fl}$***Ikbkb***$^{fl/fl}$,

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Box 1. NF-κB: A Tale of Two Pathways and Many Triggers

The NF-κB family of transcription factors are hetero- or homodimers of Rel family member proteins, of which there are five – RelA, c-Rel, p50, RelB, and p52 – that are conserved between mouse and human. The genes *Rela*, *Rel*, and *Relb* encode RelA, c-Rel, and RelB proteins, respectively, while p50 and p52 are cleavage products of p105 and p100 proteins that are encoded by the *Nfkb1* and *Nfkb2* genes. A family of inhibitory proteins, the inhibitors of κB (IκB) that include p105 and p100 [84], bind to Rel dimers and sequester them in the cytoplasm. Release of NF-κB dimers is regulated in much the same way in mouse and human. The IKK complex, a trimeric complex of two kinases, IKK1 (IKKα) and IKK2 (IKKβ) plus a regulatory subunit Nemo (IKKγ), phosphorylates IκB (P in Figure I), resulting in its degradation by the proteasome and permitting NF-κB dimers to enter the nucleus. This pathway regulates activation of RelA:p50 and c-Rel:p50 NF-κB dimers sequestered by IκBα or p105, and is termed the canonical NF-κB pathway (Figure I). The activity of RelB:p52 dimers is instead regulated by the so-called alternative NF-κB pathway. Dimers are sequestered specifically by p100 protein whose phosphorylation and degradation are specifically controlled by an IKK complex composed of an IKK1 homodimer. Activation of this IKK1 complex is instead triggered by NF-κB-inducing kinase (NIK).

Surface receptors connect with canonical NF-κB pathways by various mechanisms. However, all have in common the recruitment of E3 ubiquitin ligases that allow the creation of a ubiquitin scaffold that is essential for the recruitment and activation of the TAB1/2/TAK1 complex, in turn activating the IKK complex. Following T cell receptor triggering, PKCθ phosphorylates Card11, allowing further recruitment of BCL10 and MALT1 molecules to form the large multimeric CBM complex (reviewed in detail in [85]). Recruitment of the E3 ubiquitin ligase, tumor necrosis factor receptor (TNFR)-associated factor (TRAF)-6, results in the creation of the ubiquitin scaffold. By contrast, many TNFRSF proteins have TRAF-interacting motifs that can directly recruit TRAF proteins. In addition to TRAF6, TRAF 2, 3, and 5 also have E3 ubiquitin ligase activity [86] that creates the ubiquitin scaffold necessary to recruit TAB1/2/TAK1 and IKK complexes. Other TNFRSF members, such as TNFR1 and DR3, lack TRAF-interacting motifs and instead have death domains that allow the recruitment of adapters such as TNFR1-associated death domain protein (TRADD), that in turn can bind to and recruit TRAFs. By contrast, activation of the alternative pathway results from stabilization of NIK protein following inhibition of its degradation by TRAF2/3. In mouse T cells, expression of both *Nfkb2* and *Relb* is dependent upon intact canonical signaling [24].

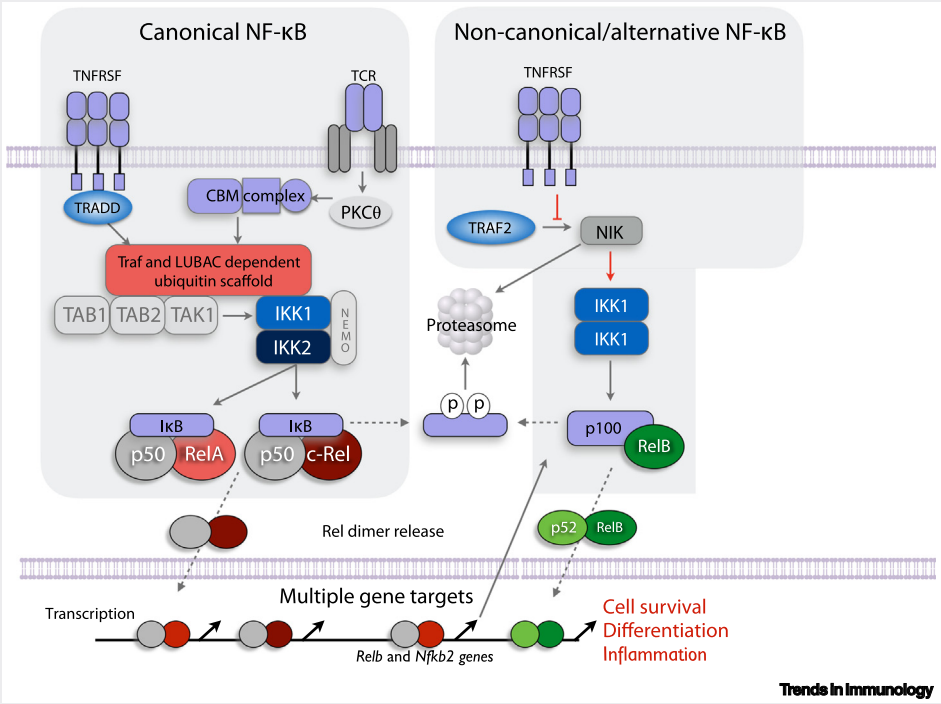

Figure I. Pathways of NF-κB Activation.

Cd4^{cre}*Map3k7*$^{fl/fl}$, or in either *Cd4*^{cre}*Hoil*$^{fl/fl}$ or *Cd4*^{cre}*Hoip*$^{fl/fl}$ mice, respectively, resulting in arrested thymocyte development at the CD4^{+} and CD8^{+} single-positive (SP) stages following **positive selection** [7–11]. The timing of arrest is not an artefact of the *Cd4*^{cre} recombinase drivers used

Glossary

A20: an early NF-κB-induced protein with ubiquitin-editing activity.
***Chuk*:** gene coding for the IKK1 component of the IKK complex.
c-Rel: canonical Rel pathway subunit encoded by the *Rel* gene.
F5: influenza virus peptide-specific T cell receptor (TCR) transgenic line.
***Gld*:** mice with a spontaneous inactivating mutation in the Fas ligand (*Faslg*) gene.
***Ikbkb*:** gene coding for IKK2 component of the IKK complex.
IKK: inhibitor of κB kinase complex whose phosphorylation targets include NF-κB, IκB repressors (targeted for degradation), and RIPK1, thereby inhibiting its kinase activity.
***Lpr*:** mice with a spontaneous inactivating mutation in the *Fas* gene.
LUBAC: ubiquitin-editing complex that uniquely attaches M1-linked linear ubiquitin chains.
MLKL: protein essential for the induction of necroptosis.
***Nfkb1*:** gene coding for the IκB protein p105.
***Nfkb2*:** gene coding for the IκB protein p100.
OT-I: ovalbumin peptide-specific TCR transgenic mouse.
p50: cleavage product of p105.
p52: cleavage product of p100.
Positive selection: thymic selection event that identifies double-positive (DP) thymocytes bearing TCRs with intermediate avidity for self-MHC complexes, and stimulates their continued development.
pre-TCR complex: heterodimer of mature TCRβ protein and invariant pre-Tα chain expressed by DN3/4 thymocytes that is required for their maturation into DP thymocytes.
RelA: canonical Rel pathway subunit encoded by the *Rela* gene.
RelB: alternative Rel pathway subunit encoded by the *Relb* gene.
RIPK1: serine/threonine kinase that functions as a ubiquitin scaffold adaptor in TNFR complex 1 but whose kinase activity also triggers apoptosis or necroptosis
Scurfy-like syndrome: a generalized autoimmune disorder in mice lacking FOXP3 expression.
TAK1: kinase required for activation of the IKK complex during NF-κB activation.
***Tnfaip3*:** gene coding for A20 protein.

Box 2. Multi-Complex Signaling by TNFR1

In mouse, ligation of TNFR1 by TNF ligands results in signaling processes that can give rise to several different signaling complexes that either result in (i) activation of NF-κB, or (ii) triggering cell death by apoptosis or by a distinct form of inflammatory cell death termed necroptosis (reviewed in [28,29]). Initial ligation of TNFR1 allows recruitment of the E3 ligase TRAF2 via TRADD, followed by the formation of a ubiquitin scaffold. Although initiated by TRAF2, further recruitment of RIPK1 together with the activity of other ubiquitin ligases – cellular inhibitor of apoptosis proteins (cIAP) and the linear ubiquitin chain assembly complex (LUBAC) – cooperate to add ubiquitin modifications both to themselves and to RIPK1, thus generating a multiprotein complex and ubiquitin scaffold (Figure I). This results in the formation of the so-called TNFR complex I, that allows TAB1/2/TAK1 and IKK complexes to be recruited and allows IKK activation by TAK1, thereby triggering subsequent NF-κB activation. The stability of complex I may be transient in the face of deubiquitinases such as A20 and CYLD. Dissolution of complex I results in the formation of one of several cell death-inducing complexes. Recruitment of Fas-associated protein with death domain (FADD), and caspase 8 by TRADD [87,88], results in formation of complex IIa that triggers apoptosis by activating caspase 8 (CASP8), whereas additional recruitment of RIPK1 forms complex IIb that is a potent inducer of apoptosis [89–91]. In the absence of caspase 8 protease activity, RIPK3 and MLKL (mixed lineage kinase domain-like pseudokinase) are recruited to complex IIb and together trigger necroptosis, that is also RIPK1 kinase-dependent (reviewed in [28,29]). The utilization of RIPK1 as an adapter during TNFR1 signaling is probably no accident. As a component of complex I, RIPK1 comes into close proximity with the IKK and TAB1/2/TAK1 complexes. Recent studies reveal that phosphorylation (P in Figure I) of RIPK1, by IKK and TAK1, represses RIPK1 kinase activity [1,33], preventing triggering of cell death. Indeed, blocking IKK or TAK1 activity is sufficient to sensitize many cells to TNF-induced cell death. Importantly, this protective function of RIPK1 phosphorylation can occur independently of NF-κB signaling [1,24]. These studies reveal the polyfunctionality of such kinases, and how they regulate cell fate decisions – not only as upstream activators of NF-κB signaling but also through targeting other substrates in the signaling pathway, such as RIPK1, to block cell death induction.

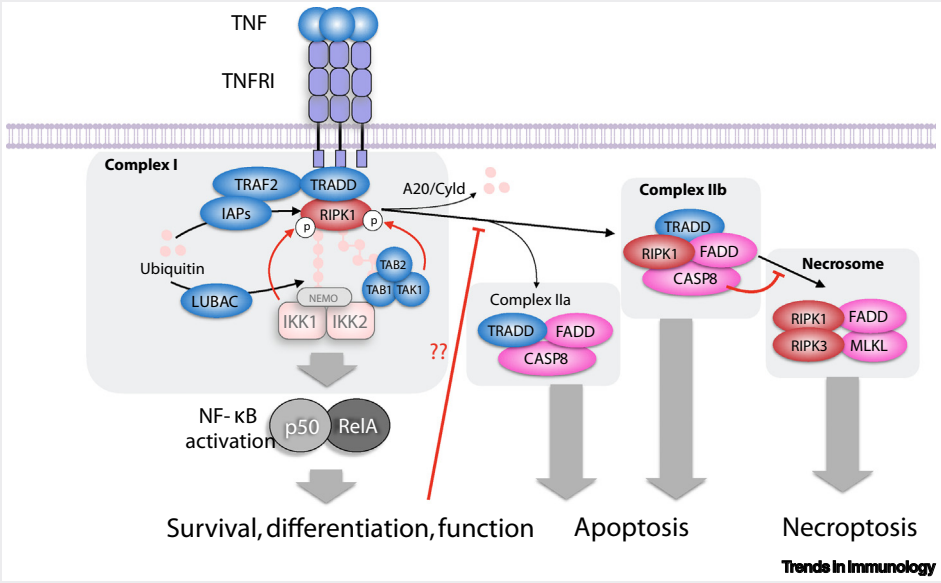

Figure I. Structure and Relationships of Different TNFR1 Signaling Complexes.

to delete genes in DP thymocytes because more recent studies, in which IKK1/2 genes were deleted earlier in development in double-negative progenitors in *huCD2*^{icre}*Chuk*$^{fl/fl}$*Ikbkb*$^{fl/fl}$ mice, report an identical block at the SP stage, and normal development before this stage [12].

SP thymocytes undergo a phase of maturation lasting many days [13,14], during which time they acquire receptors that are necessary for normal recirculation, such as L-selectin, CCR7, and S1P1, as well as the ability to proliferate in response to TCR triggering [11]. Transcriptional analysis of SP mouse thymocytes reveals a prominent NF-κB gene signature specifically in late-stage SP thymocytes, supporting the idea that maturation is dependent upon NF-κB (Figure 1) [11,12].

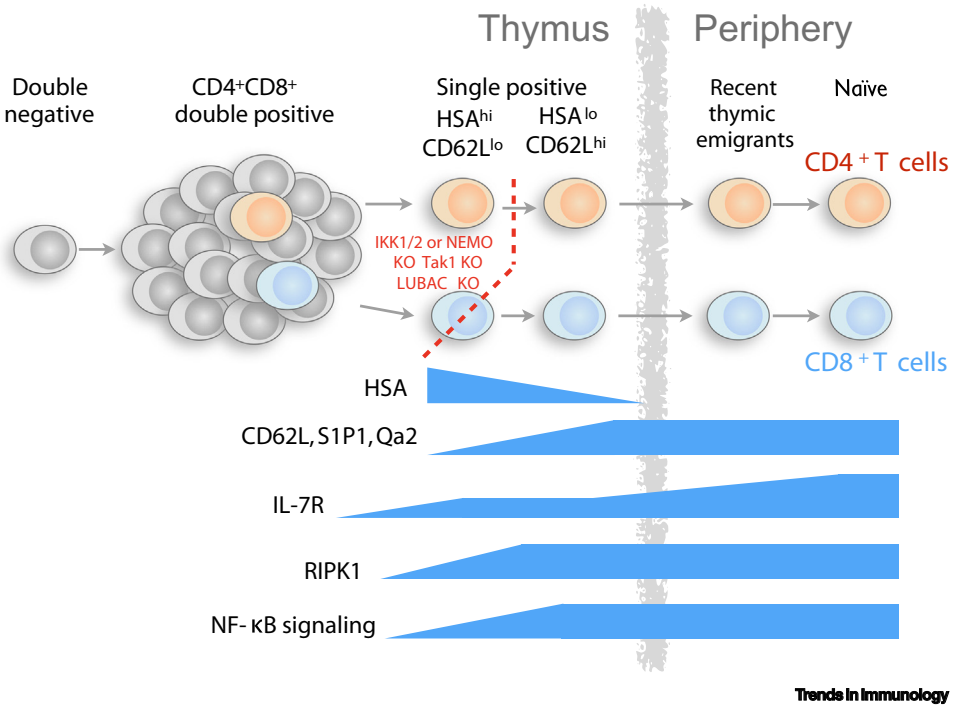

Figure 1. NF-κB and Cell Death Signaling during Mouse Thymic Development. Following positive selection, newly generated CD4$^+$ or CD8$^+$ single-positive (SP) thymocytes undergo a maturation process characterized by loss of HSA (CD24) expression and acquisition of CD62L, S1P1, and IL-7R expression [11,12]. The acquisition of NF-κB signaling competency and susceptibility to TNF-induced death signaling also appears to be part of this maturation process because SP thymocytes also induce RIPK1 expression and the ability to transmit TNF receptor superfamily (TNFRSF) signals [24]. Although the development of conventional αβ T cells continues unimpeded despite loss of Rel subunits, loss of IKK, TAK1, or LUBAC function results in a profound block in thymopoiesis during SP thymocyte development (red dotted line). In the case of TAK1 and IKK deficiencies, this is caused by unopposed TNF-induced cell death [11,12]. Blue tapers indicate expression of the corresponding markers. Abbreviation: KO, knockout

Although the TCR has been considered to be the key upstream trigger of NF-κB activity in thymocytes, NF-κB-induced genes are most evident among late-stage mouse SPs [11,12] (Figure 1), at a point when thymic development is no longer under the influence of TCR signaling [15]. Furthermore, it is notable that the CARD11/BCL10/MALT1 (CBM) complex, that links TCR signaling to NF-κB activation (see Figure I in Box 1), is redundant for T cell selection [16–22], further arguing against a role for TCR-triggered NF-κB in thymic development. Recent studies have shed light on this puzzle by revealing that the block in SP development is in fact the result of cell death triggered by TNF signaling. Specifically, the apparent developmental block in IKK1/2- or TAK1-deficient T cells, in *huCD2*^{icre}*Chuk*$^{fl/fl}$*Ikbkb*$^{fl/fl}$ and *Cd4*^{cre}*Map3k7*$^{fl/fl}$ mice, is overcome *in vivo* by genetic ablation of TNF or TNFR1, or by TNF-blocking monoclonal antibodies (mAbs) [11,12]. TNFR1 signaling can trigger cell death as well as NF-κB activation (Box 2) and, in the absence of key triggers of NF-κB, such as the IKK complex or TAK1, death becomes the default cell fate. The rescue of SP development by TNF blockade in IKK1/2- or TAK1-deficient T cells has also revealed that NF-κB signaling is not required for continued development of SP thymocytes. Confirming this, recent studies of *Cd4*^{cre}*Rel*$^{fl/fl}$ *Rela*$^{fl/fl}$ and *Cd4*^{cre}*Rela*$^{fl/fl}$ *Nfkb1*$^{-/-}$ *Rel*$^{-/-}$ mice, whose T cells lack **RelA** and **c-Rel**, did not find any developmental block of conventional αβ T cells in the thymus [23,24]. In mouse embryonic fibroblasts (MEFs) and cell lines, *Relb* and ***Nfkb2*** expression depends upon canonical NF-κB signaling [25–27] (Box 1). This is also the case in thymocytes because expression of both genes is lacking in RelA/c-Rel-deficient mouse thymocytes [24].

Therefore, progression of conventional αβ T cell development proceeds in a relatively normal manner in the complete absence of either canonical or alternative NF-κB pathways.

Thymocyte Survival Is Controlled by IKK Independently of NF-κB

At first sight, the finding that NF-κB-deficient thymocytes undergo relatively normal development appears to be at odds with the block in SP thymic development observed in mice lacking upstream triggers such as IKK, TAK1, and LUBAC [7,10–12]. The cell death phenotype observed in these studies was presumed to result from the absence of a protective NF-κB survival signal to oppose TNF-induced death signaling. In fact, in contrast to IKK1/2-deficient thymocytes, canonical Rel subunit-deficient thymocytes from *Cd4*^{cre}*Rela*$^{fl/fl}$*Nfkb1*$^{-/-}$*Rel*$^{-/-}$ mice were resistant to TNF-induced cell death *in vitro* [24]. This apparent contradiction with the earlier studies was reconciled by work revealing that MEFs from *Ikbkb*$^{-/-}$ mouse embryos lacking IKK2 undergo cell death in two phases – slow and fast – that are respectively dependent and independent on NF-κB signaling [1]. Although loss of NF-κB transcriptional activity (by inhibiting protein translation with cycloheximide) did result in a slow cell death response, the fast cell death response was blocked by the **RIPK1** inhibitor necrostatin 1 (Nec1), and was therefore dependent on RIPK1-triggered apoptosis or on an alternative form of cell death termed necroptosis (reviewed in [28,29]). RIPK1 is a crucial component of death-inducing complexes that form following TNF stimulation (see Figure I in Box 2) [30]. In this context, the IKK complex blocks cell death by phosphorylating RIPK1 and thus inhibiting its kinase activity [1]. RIPK1 phosphorylation is a key cell death checkpoint in other cells and is mediated by various kinases, including IKK and TAK1 [31]. In developing thymocytes, RIPK1 is a central regulator of TNF receptor superfamily (TNFRSF) signaling. Cell death in the absence of IKK1/2 in *huCD2*^{icre}*Chuk*$^{fl/fl}$*Ikbkb*$^{fl/fl}$ mice is RIPK1-dependent because IKK1/2-deficient thymocytes can be rescued from TNF-induced cell death by blocking RIPK1 kinase activity, either by genetic mutation in *Ripk1*^{D138N} mice or by using the kinase inhibitor Nec1 *in vitro* [24]. This protective function of RIPK1 phosphorylation can occur independently of NF-κB signaling [1], and the resistance of RelA/c-Rel-deficient thymocytes from *Cd4*^{cre}*Rela*$^{fl/fl}$***Nfkb1***$^{-/-}$*Rel*$^{-/-}$ mice to TNF-induced cell death *in vitro* is dependent upon IKK kinase activity, revealing that thymocytes can control TNF-induced cell death exclusively by this route [24]. These studies reveal the polyfunctionality of the IKK complex and how it regulates cell fate decisions – not only as an upstream activator of NF-κB signaling but also by targeting other substrates in the signaling pathway, such as RIPK1, thus blocking cell death induction.

RIPping through Development

The central role of RIPK1 in activating NF-κB and triggering cell death processes may also account for the specific susceptibility of maturing SP thymocytes to cell death in the absence of regulators such as IKK, TAK1, and LUBAC. RIPK1 expression is negligible in mouse DP thymocytes, but undergoes substantial transcriptional upregulation following positive selection, reaching a maximal level in mature SPs, confirmed by protein expression [24] (Figure 1). This may contribute to explaining why NF-κB-regulated gene induction and susceptibility to cell death coincide in the same mature SP populations. Immature DP and HSA^{hi}CD4^{+} SP thymocytes from wild-type (WT) mice that do not express a high abundance of RIPK1 are weakly susceptible to TNF-induced cell death that is RIPK1-independent [24]. It appears that RIPK1 induction in more mature SP populations protects thymocytes from this form of complex IIa-mediated cell death (see Figure I in Box 2), provided that its kinase activity is kept in check by the activity of IKK1/2. In thymocytes, RIPK1 expression might stabilize the formation of complex I (see Figure I in Box 2) following TNF stimulation. Whether RIPK1 mediates cell death of mature SPs lacking TAK1 or LUBAC remains to be determined. TAK1 is necessary to activate the IKK complex in HeLa cells [32], and can directly phosphorylate RIPK1 in the L929 mouse fibroblast cell line [33], whereas LUBAC ubiquitination activity is important for recruiting IKK to complex I [34–36] and therefore into

proximity with RIPK1. In mice lacking T cell expression of TAK1 (*Cd4*^{cre}*Map3k7*$^{fl/fl}$ mice) or LUBAC (*Cd4*^{cre}*Hoil*$^{fl/fl}$ mice), the expression of constitutively active IKK2 constructs was used to reconstitute NF-κB activation, and this provided some degree of rescue of thymocyte development *in vivo* [10,11]. However, although unappreciated at the time, the ability of IKK2 to phosphorylate RIPK1 might be more relevant than triggering downstream NF-κB.

NF-κB Survival Programs at the Heart of Peripheral T Cell Homeostasis

Although NF-κB does not appear to play an instructive role in guiding the thymic development of conventional αβ T cells [24], it is nevertheless important for the normal homeostasis and function of peripheral T cells. Transcriptional analysis of mouse thymocyte subsets reveals a strong NF-κB-induced gene signature in mature SP thymocytes that is not apparent at earlier developmental stages [11,12,24], and this targets a large number of genes involved in cell survival, including cIAPs, IL-7R, and also interferon (IFN) signaling (Figure 1). The functional significance of these gene targets for naïve T cell function has, in most cases, not yet been tested. IFN signaling regulated by NF-κB activity appears to be important for phenotypic maturation of thymocytes because *Ifnar1*$^{-/-}$ mouse thymocytes lack expression of nonclassical MHC molecule, Qa2 [11]. The key IL-7R survival pathway is regulated by NF-κB in naïve T cells: IL-7R is essential throughout T cell development, except for positive selection – where DP thymocytes lose IL-7R expression to ensure that their developmental progression is governed solely by TCR signaling [37]. Resumed IL-7R expression is therefore an important maturation step in post-selection thymocytes to ensure long-term survival of new T cells [38]. Re-expression of *Il7r* requires TCR signaling during positive selection because IL-7R expression on SPs correlates with TCR affinity for self-peptide MHC in **F5** and **OT-I** TCR transgenic mouse strains. Continued expression in SPs depends upon both the transcription factor FOXO1 and NF-κB because *Il7r* is lost in SPs from both *Cd4*^{cre}*Foxo1*$^{fl/fl}$ and *Cd4*^{cre}*Rela*$^{fl/fl}$*Rel*$^{-/-}$ mice [24,39]. Indeed, validated NF-κB binding sites are present in the IL-7R gene (*Il7r*/*IL7R*) promoters in both mouse and human [40]. TCR signaling does not appear to be the trigger for NF-κB signaling in this process because TCR-dependent induction of IL-7R occurs immediately following positive selection and does not require NF-κB [38]. Instead, TNFRSF signaling is at least one trigger because ligation of either TNFR1 and CD27 *in vitro* can induce IL-7R expression in SP thymocytes from F5 TCR transgenic mice [38] (Figure 2). However, IL-7R expression and peripheral T cell numbers are normal in *Tnfrsf1a*$^{-/-}$ and *Cd27*$^{-/-}$ mice [12,41]), and this is relevant because it suggests that there can be redundancy between TNFRSF members for stimulating NF-κB activity in developing T cells [38]. However, other triggers of NF-κB signaling in these cells remain to be identified (Figure 2).

Transmission of NF-κB signaling by TNFRSF members is also dependent upon RIPK1 for optimal signaling in thymocytes [24] (see Figure I in Box 2); therefore, developmental control of RIPK1 expression also contributes to the stage-specific induction of IL-7R in thymocytes. The NF-κB factor driving this aspect of thymocyte maturation also appears to be largely RelA-dependent because both CD4^{+} and CD8^{+} naïve T cell numbers, and IL-7R expression in *Nfkb2*$^{-/-}$ mice that lack **p52** Rel subunit or *Rel*$^{-/-}$*Nfkb1*$^{-/-}$ mice that lack **p50** and c-Rel, are essentially normal [24,40,42]. By contrast, peripheral naïve T cell numbers in *Cd4*^{cre}*Rela*$^{fl/fl}$*Nfkb1*$^{-/-}$ mice are substantially reduced [24]. Nevertheless, there is some redundancy between c-Rel and RelA because gene ablation of both factors in *Cd4*^{cre}*Rela*$^{fl/fl}$*Nfkb1*$^{-/-}$*Rel*$^{-/-}$ mice results in greater loss of peripheral naive T cell numbers than in *Cd4*^{cre}*Rela*$^{fl/fl}$*Nfkb1*$^{-/-}$ mice [24]. It is unclear whether these activities reflect distinct gene-targeting specificities between RelA- and c-Rel-containing dimers or are merely a consequence of differing relative expression levels of these units in naïve CD4^{+} and CD8^{+} T cells.

The profound T cell deficiency in *huCD2*^{icre}*Chuk*$^{fl/fl}$*Ikbkb*$^{fl/fl}$ mice lacking IKK1/2, and in *Cd4*^{cre}*Map3k7*$^{fl/fl}$ mice lacking TAK1, cannot be reversed by blocking TNF signaling or RIPK1

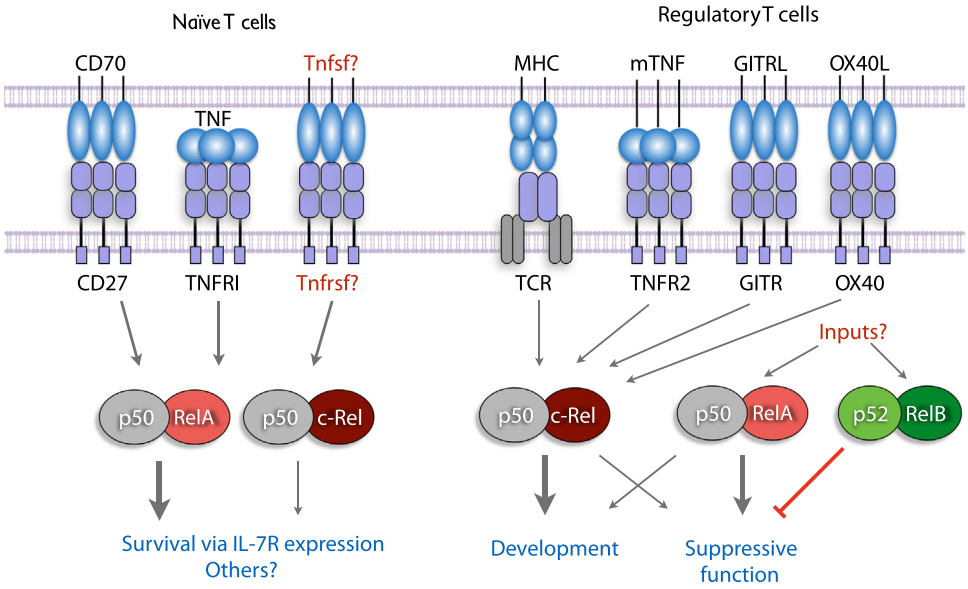

Figure 2. Contrasting Roles of Upstream Triggers and NF-κB Subunit Involvement in the Development and/or Maintenance of Conventional versus Regulatory T Cells (Tregs) in Mice. During maturation of newly generated conventional naïve T cells, RelA- (and to a lesser extent c-Rel)-containing NF-κB dimers induce the expression of a range of genes that are required for normal naïve T cell homeostasis [24]. The alternative NF-κB pathway appears to be redundant for naïve T cell development. Signaling is driven by TNF receptor superfamily (TNFRSF) members TNFR1 and CD27, although there appears to be redundancy with other, so far unidentified, triggers (indicated by Tnfsf?/Tnfrsf?). Treg development relies upon both T cell receptors (TCRs) and TNFRSF members to trigger a primarily c-Rel-dependent NF-κB activity that is essential for their intrathymic development [21,60]. In mature Tregs, RelA-dependent NF-κB activity is essential for normal suppressive function, whereas activation of alternative NF-κB signaling inhibits their suppressive function [23,48,51,57,58], although the precise upstream receptors for these activities remain to be identified (indicated by Inputs?).

kinase activity with kinase-dead mutant RIPK1^{D138N} [11,12,24]. NF-κB signaling in mature thymocytes is essential to achieve full homeostatic potential [38,40]. However, whether such signaling remains a constitutive requirement for survival of recirculating naïve T cells is unclear. Tamoxifen-induced ablation of IKK2 in mature F5 TCR transgenic T cells in F5 *Rag1*$^{-/-}$*Rosa26*^{creERT}*Ikbkb*$^{fl/fl}$ mice does not appear to impair their survival or homeostatic proliferation upon adoptive transfer into *Rag1*$^{-/-}$ lymphopenic hosts, even though such deficiency does prevent normal NF-κB signaling in mature thymocytes [38]. However, other studies find that re-expression of IL-7R by T cells from *Cd4*^{cre}*Ikbkb*$^{fl/fl}$ mice, that have lost expression following *in vitro* culture with IL-7, does require IKK2 expression [40]. Therefore, fine tuning of IL-7R expression by T cells to regulate their homeostasis [37] represents at least one survival function of NF-κB in mature naïve T cells.

The Reins on Regulation

In contrast to conventional αβ T cells, NF-κB signaling is fundamental in guiding the development of non-conventional T cell populations in the thymus. Both the development and function of FOXP3^{+} Tregs are crucially reliant on NF-κB. Indeed, profound deficits in intrathymic development of Tregs have long been noted in c-Rel-deficient *Rel*$^{-/-}$ mice [42–45] and T cell-targeted knockouts of IKK1 and IKK2 components in *CD4*^{cre}*Chuk*$^{fl/fl}$ and *Cd4*^{cre}*Ikbkb*$^{fl/fl}$ mice, respectively [21,46]. Recent studies highlight a complex regulation of Treg biology involving multiple receptors and contrasting roles for the different Rel subunits. Specifically, NF-κB signaling during Treg development appears to be mediated by canonical rather than alternative pathways because ablation of genes encoding **RelB**, p100, or upstream activator NIK (Box 1) by either *Cd4*^{cre} or

Foxp3^{cre} does not affect thymic Treg numbers [47–50]. Although *Cd4*^{cre}-mediated deletion of *Rela* causes only a modest reduction in thymic Treg numbers [23,24,51], there is some redundancy between c-Rel and RelA because complete loss of Treg numbers only occurs with their combined loss in *Cd4*^{cre}*Rela*$^{fl/fl}$*Rel*$^{fl/fl}$ mice [23,24]. The development of thymic Tregs occurs by two different pathways, via either CD25^{+}FOXP3^{-} or CD25^{-}FOXP3^{+} precursors [52], followed by IL-2- and IL-15-dependent maturation to a mature CD25^{+}FOXP3^{+} phenotype [53–55]. CD4SP thymocytes in *Rel*$^{-/-}$ mice lack both these precursor populations [23,56]. Therefore, c-Rel appears to be required for optimal Treg development from both CD25^{+}FOXP3^{-} and CD25^{-}FOXP3^{+} precursors.

Rel subunit preference during development does not extend to Treg survival or function, implying that NF-κB has distinct functions in these different processes. Genetic ablation of RelA or c-Rel after commitment to the Treg lineage using *Foxp3*^{cre} drivers to delete *Rela*$^{fl/fl}$ or *Rel*$^{fl/fl}$ alleles reveals that Tregs persist in the absence of either RelA or c-Rel, or of both subunits, albeit at reduced frequencies relative to normal WT mice [23]. Although *Foxp3*^{cre}*Rel*$^{fl/fl}$ mice remain healthy, ablation of RelA in *Foxp3*^{cre}*Rela*$^{fl/fl}$ mice results in autoimmunity and death [23,51,57,58]. Moreover, RelA appears to be required for stable *Foxp3* expression and for generating the Treg effector phenotype [51,58]. In addition, c-Rel is important for suppressive Treg functions in some scenarios: specifically, in adoptive transfer mouse models of inflammatory bowel disease, in which Treg cell numbers are limiting, c-Rel-deficient Tregs from *Foxp3*^{cre}*Rel*$^{fl/fl}$ donors failed to control naïve T cell-induced disease [23]. In addition, combined loss of c-Rel and RelA expression induced by *Foxp3*^{cre} resulted in more profound disease manifestations than in *Foxp3*^{cre}*Rela*$^{fl/fl}$ mice, namely runting and a **scurfy-like syndrome** [23].

Although alternative NF-κB signaling in T cells appears to be dispensable for Treg development, recent studies do suggest that it plays a role in Treg maintenance and function (Figure 2). In the complete absence of alternative NF-κB signaling, thymic Treg numbers were normal in bone marrow chimeras hosting a NIK-deficient hematopoietic system, as well as in *Cd4*^{cre}*Nik*$^{fl/fl}$ mice, but peripheral Treg numbers were reduced relative to WT mice [47,59]. In contrast to these findings, Treg numbers were unaffected when only *Relb* was deleted in *Cd4*^{cre}*Relb*$^{fl/fl}$ or *Foxp3*^{cre}*Relb*$^{fl/fl}$ mice [48,50]. Moreover, loss of the p100 NF-κB inhibitor in Tregs of *Foxp3*^{cre}*Nfkb2*$^{fl/fl}$ mice resulted in a RelB-dependent expansion of Tregs that was also associated with the development of inflammatory disease, suggestive of defective Treg function in these mice [48]. Therefore, it appears that normal Treg homeostasis depends upon the restraint of RelB by p100, and that excessive RelB activity is in fact deleterious to Treg function.

Matching the complexity of NF-κB function in Tregs, upstream receptor triggers of NF-κB activity that are necessary for Treg development are also diverse, including TCR and TNFRSF members (Figure 2). Thymocytes whose TCRs have a high avidity for self are preferentially selected into the Treg lineage [2]. In contrast to selection of naïve T cells, TCRs of Treg progenitors appear to mediate a pivotal activation of NF-κB because uncoupling the TCR from downstream NF-κB activation in *Bcl10*$^{-/-}$ or PKCθ-deficient *Prkcq*$^{-/-}$ mice profoundly inhibited Treg development [21]. In addition, the emergence of FoxP3^{+} thymic Tregs required costimulation through TNFRSF members OX40, GITR, and TNFR2. In this study, mature Treg numbers were modestly reduced in *Tnfrsf18*$^{-/-}$ and *Tnfrsf4*$^{-/-}$ mice lacking GITR and OX40, respectively [60]. However, combined blockade of OX40, GITR, and TNFR2 with mAbs in fetal thymic organ cultures *in vitro* abrogated almost all Treg development [60]. Thus, TCR activation of NF-κB might represent a feedforward loop in Treg development, given that optimal expression of these specific TNFRSF members depends upon TAK1 signaling [60]. TNFRSF receptors also play an important role in mature Tregs. Peripheral Treg proliferation *in vivo* in response to IL-2 was enhanced by costimulation with OX40 and GITR ligands, and the authors suggest that this is important for

generating effector Tregs in peripheral tissues [57]. Thus, a constitutive requirement for NF-κB signaling in Treg cells might continue to be mediated by the same TNFRSF members that facilitate their development, although further experiments to fully address this point are warranted.

The role of death signaling in Treg homeostasis is unclear. Deletion of genes encoding regulators of cell death such as IKK2, TAK1 or LUBAC in $CD4^{cre}Ikbkb^{fl/fl}$ [21], $Foxp3^{cre}Map3k7^{fl/fl}$ [61], and $Cd4^{cre}Hoil^{fl/fl}$ [10] mice results in reduced numbers of peripheral Tregs. It is unclear whether a failure to directly control death signaling contributes to the absence of Treg development in these mice. There is, however, some evidence that regulation of death signaling may be required for long-term maintenance of mature Tregs given that $Foxp3^{cre}$-mediated deletion of *Ikbkb*, *Map3k7*, or *Hoip* results in a greater loss of peripheral Treg than does the combined ablation of both c-Rel and RelA in $Foxp3^{cre}Rela^{fl/fl}Rel^{fl/fl}$ mice [23]. The phenotype of IKK2- or LUBAC-deficient Tregs may therefore reflect a compound phenotype resulting from loss of both NF-κB activation and control of extrinsic cell death pathways, although this requires formal testing.

Activation and Beyond

The key role of NF-κB signaling for activation of T cells following TCR triggering has long been appreciated (reviewed in [62]). Thymocytes deficient in upstream kinases such as IKK fail to divide in response to TCR stimuli *in vitro* [11,24], whereas mice whose T cells lack expression of c-Rel fail to develop memory-phenotype populations *in vivo* [24,42]. This is consistent with evidence that NF-κB signaling plays essential functions in c-Myc-dependent blast transformation of mouse T cells [63,64] and in blast survival. In both mouse and human activated T cells, the expression of a dominant negative IκB construct by a retrovirus vector or by *Lck* promoter-driven transgenesis results in impaired BclxL expression [65,66]. However, the importance of this expression is questioned by the observation that T cell-specific ablation of BclxL in $Lck^{cre}Bcl2l1^{fl/fl}$ mice does not result in activated T cell death [67]. Furthermore, because addition of exogenous IL-2 can rescue defective $Rel^{-/-}$ T cell responses to CD3 crosslinking *in vitro*, optimal IL-2 synthesis by activated T cells is NF-κB-dependent and promotes their survival [68]. Thus, NF-κB induces a broad inflammatory gene response in many cell types, and cooperation with chromatin-remodeling factors plays an important role in determining the spectrum of target genes (reviewed in [69]), which is also likely to be the case in T cells.

The more recent discovery of alternative modes of cell death has started to reveal an additional layer of complexity in the control of cell death in activated T cells. Studies of whole mouse knockouts show that caspase 8 plays an essential role in protecting cells from necroptosis via cleavage of RIPK1, RIPK3, or CYLD, thus preventing formation of the necroptosome [70–72] (see Figure I in Box 2). Analysis of these pathways in T cells reveals further evidence of careful developmental orchestration of death signaling. Fas-induced T cell apoptosis is dependent upon caspase 8 because T cells from $Lck^{cre}Casp8^{fl/fl}$ mice are resistant to anti-Fas-induced cell death *in vitro* [73]. ***Lpr*** and ***Gld*** mice that have defective Fas signaling exhibit extensive lymphoproliferative disease [74]. By contrast, T cell-specific deletion of *Casp8* using $Cd4^{cre}$ [75] or $pLck^{cre}$ [73] either has no effect on peripheral T cell numbers or results in reduced T cell numbers, respectively, with no impact upon thymic development in either case. However, *Casp8*-deficient T cells from $Cd4^{cre}Casp8^{fl/fl}$ OT-I mice fail to respond to ovalbumin stimulation either *in vitro* or *in vivo*, and instead undergo cell death that can be blocked by RIPK1 inhibitor Nec1 [75]. Ablating *Ripk3* in $Cd4^{cre}Casp8^{fl/fl}$ mice confirms the necroptotic nature of this T cell death *in vivo* because $Ripk3^{-/-}Cd4^{cre}Casp8^{fl/fl}$ mice develop a lymphoproliferative disease, with outgrowth of $CD4^{-}CD8^{-}$ double-negative T cells comparable to that observed in *Lpr* or *Gld* mice [76]. Together, these studies show that T cells are susceptible to necroptosis and that caspase 8 actively blocks this cell fate *in vivo*.

Necroptosis and NF-κB activation also appear to be subject to crossregulation in activated T cells in much the same way that RIPK1 regulates both apoptosis and NF-κB activation in mature thymocytes [24]. In the former case, the anti-inflammatory protein **A20** appears to be the key link in these processes because it can restrict both NF-κB signaling and necroptosis. Specifically, A20 is a deubiquitinating enzyme that has complex activities, including the removal of lysine 63 (K63)-linked polyubiquitin chains [77] (that is essential for the formation and maintenance of complex I); it can also target proteins for degradation by building K48-linked ubiquitin chains [77,78]. In T cells, loss of A20 expression by $Cd4^{cre}$-targeted deletion of ***Tnfaip3*** has no obvious impact on the development of conventional T cells, but results in increased Treg populations that correlate with enhanced NF-κB activation in T cells [79] – an outcome consistent with an anticipated deregulation of NF-κB. However, anti-CD3-stimulated, A20-deficient T cells from $Cd4^{cre}Tnfaip3^{fl/fl}$ mice exhibit survival defects *in vitro*, particularly in the presence of caspase inhibitors, that are rescued by *Ripk3* deficiency, suggesting a role for A20 in regulating necroptosis [80]. Therefore, RIPK1–RIPK3 complex formation depends upon ubiquitination, and RIPK3 appears to be a target of A20-mediated deubiquitination that restricts the formation of the necrosome and induction of cell death.

The observation that necroptosis of T cells from $Cd4^{cre}Casp8^{fl/fl}$ mice only occurs following T cell activation implies that this cell fate is restricted to antigen-experienced T cells. This is a consequence of *Mlkl* induction during T cell activation because gene expression analysis of naïve mouse T cells reveals expression of other key components, such as RIPK1 and RIPK3, but not of **MLKL** [24]. Therefore, it appears that either naïve T cells are protected from necroptosis by the absence of MLKL or that activated T cells require some type of defense mechanism such as that offered by necroptotic cell death. Thus, further testing is warranted to better elucidate these pathways. Whichever the case, these findings represent another example of how cell death processes in T cells are under strict developmental control, suggestive of a specific evolutionary adaptation, but many of the resulting functional outcomes remain to be fully elucidated.

Outstanding Questions

Is tonic NF-κB signaling required for long-term survival of naïve T cells?

Do developmental NF-κB gene programs contribute to the functional maturation of conventional T cells?

Does RIPK1-dependent control of death signaling contribute to maintenance of Tregs?

Why do conventional T cells become susceptible to necroptosis following antigen experience, and what (patho) physiological function does this play during immune responses?

Does NF-κB signaling contribute to long-term homeostasis of memory T cell populations?

How do NF-κB and cell death pathways combine to control the development and homeostasis of nonconventional T cells such as γδ, iNKT, MAIT, and IEL T cells?

Concluding Remarks

As highlighted here, NF-κB and death signaling are intertwined through mammalian T cell development and function. The recognition that cell death is regulated by proteins such as IKK and TAK1 – that were previously thought to solely regulate NF-κB activation – raises questions about the functional roles of NF-κB in T cells. The dogma that cell death signaling is counterbalanced by prosurvival NF-κB signaling is clearly an oversimplification. However, in T cells, key components of death and NF-κB signaling, such as A20, cIAP1, and cIAP2 (Box 2), are themselves NF-κB targets [12,24], suggesting that one function of NF-κB signaling may be to tune cell death signaling under particular (but so far unidentified) conditions. Although NF-κB signaling is redundant for the march of T cell development, distinctive NF-κB-driven gene signatures are expressed in developing T cells [11,12]. IL-7R and type I IFN signaling are at least two targets of signaling [11,12], but whether other aspects of T cell function are dependent upon this NF-κB-induced program remains unclear. Given the obligate role of NF-κB in T cell activation, it is unsurprising that memory cells fail to develop in mice with defective NF-κB signaling [7,24,42]. Therefore, a key emerging question concerns whether long-term maintenance of either recirculating memory cells or tissue-resident memory depends upon continued NF-κB and/or death signaling. Most studies have focused on conventional T cells, but it is likely that NF-κB pathways also control the development and function of non-conventional T cell populations. For example, in mouse, γδ T cell development depends upon TNFRSF member CD27 [81], whereas so-called $CD8^+$ virtual memory cells [82] are specifically expanded in $Nfkb1^{-/-}$ mice [83], suggesting that NF-κB pathways play key roles in the development and homeostasis of these cells. For other populations, such as intraepithelial T cells, little is currently known, but

Box 3. NF-κB Pathways in Human Primary T cells

The accessibility of *in vitro* cell systems such as immortalized T cell lines and cultures of activated primary T cells has allowed characterization of NF-κB function during human T cell activation in parallel with mouse studies. Such studies confirm the strong similarities between how mouse and human T cells utilize NF-κB pathways during TCR-triggered activation. By contrast, much less is known about how NF-κB and death pathways regulate T cell development and homeostasis in humans. Studies on patients with primary immunodeficiencies (PIDs) provide some clues and have identified several cases in which common variable immunodeficiency (CVID) is caused by mutations in NF-κB pathway components. Such mutations are present in individuals either as homozygous mutations or as autosomal dominant mutations (haploinsufficiency) in which patients harbor a single defective allele. Although clinical presentation is likely to be a complex phenotype involving many cell types and tissues, such patients can provide important clues to how NF-κB and death signaling function in human T cells *in vivo*. Mutations in *NFKB1* have been identified as the most common single-gene cause of CVID in Europeans [92,93]. B cell-mediated immunity is profoundly affected in such individuals, although a comprehensive study of T cell function is outstanding. Cases of *RELA* haploinsufficiency have also been described, resulting in autosomal dominant, mucocutaneous ulceration, although the contribution of T cells to the inflammatory process, and whether these exhibit functional defects, has not been assessed [94]. Patients lacking LUBAC components have also been identified with inflammatory disease and immunodeficiency, although T cell function characterization has not yet been performed [95,96]. Loss of c-Rel in immunodeficient patients resulted in peripheral T cell features similar to those described in mice, with normal or above average numbers of total $CD4^+$ and $CD8^+$ T cells but greatly reduced memory cells and defective activation [97]. Similarly, T cells in patients lacking IKK2 (due to mutations in the *IKBKB* gene) almost exclusively have a naïve phenotype, fail to proliferate *in vitro*, and lack Tregs in some patients [98], much like T cell-specific mouse knockouts [7]. Patients with biallelic *RIPK1* mutations have also been identified and present with severe immunodeficiency, arthritis, and gut inflammation [99,100]; as in RIPK1-deficient mice, they are T-lymphopenic. The *Il7r* gene has been identified as a key NF-κB target in mouse T cells that is responsible for regulating homeostasis [38,40]. Although it is difficult to study T cell homeostasis in such patients, the *IL7R* genes of mice and humans have conserved NF-κB binding sites, and inhibition of NF-κB signaling in human T cells interferes with *IL7R* expression [40]. Therefore, it is tempting to speculate that IL-7R regulation might be tuned in human T cells similarly to in mice.

given the importance of NF-κB and death signaling in conventional T cells and Tregs, highlighted here, these pathways are likely important in some aspects of the biology of these cell types. Thus, detailed dissection of NF-κB and cell death pathways in such populations will be important areas of future investigation (see Outstanding Questions). Finally, mouse genetics has proved to be a powerful tool in dissecting some of these pathways *in vivo*, for example in rodents. To fully exploit this knowledge for therapeutic benefit, it will be important to determine whether NF-κB and cell death pathways function in a comparable manner in human T cells (Box 3). This may not only lead to a better understanding of the possible consequences of pharmacological interference with these pathways in normal immune function in humans, but may also reveal novel targets that can either impede T cell activity in autoimmune settings or augment adaptive immunity in the context of cancer immunotherapies.

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