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NF- κ B inducing kinase: a key regulator in the immune system and in cancer

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Abstract

NF- κ B inducing kinase (NIK) is a kinase that activates the canonical and non-canonical NF- κ B pathways to control transcriptional expression of certain proteins such as cytokines, chemokines and NF- κ B signaling molecules. Many advances have been made in understanding the molecular mechanisms by which the stability of NIK is regulated to affect downstream signaling. Genetic mouse models suggest that NIK has an essential role in regulation of the immune system as well as in the bone microenvironment. Increasing evidence links NIK to the tumorigenesis of hematological cancers, such as multiple myeloma, and solid tumors, such as pancreatic carcinoma and melanoma. Understanding the mechanism by which NIK is de-regulated will potentially provide therapeutic options for certain diseases such as autoimmunity and cancer.

Keywords

NF- κ B inducing kinase (NIK); NF- κ B; IKK; canonical pathway; non-canonical pathway

The nuclear factor- κ B (NF- κ B) family of transcription factors is comprised of a collection of structurally related proteins that modulate multiple physiological processes, ranging from immune responses to cell death and survival. These transcription factors are ubiquitously expressed and control the expression of a significant number of genes. Multiple kinases and signaling molecules are responsible for the precise regulation of the NF- κ B pathway, one of which is NF- κ B inducing kinase (NIK). In this review, we will discuss the function and regulation of NIK in relation to its role in the NF- κ B pathway, as well as some functions of NIK that are independent of the NF- κ B pathway. We will also review the functional significance of NIK in the context of physiological processes such as immune regulation, and in disease models such as autoimmunity and cancer.

1. NIK in the regulation of NF- κ B

1.1. NIK as a kinase of the canonical and non-canonical NF- κ B pathways

The family of NF- κ B in mammals is comprised of five members; p65 (RelA), p105/p50, RelB, p100/p52 and c-Rel. Regulation of NF- κ B can be achieved by two distinct pathways.

Conflicts of interest

The authors do not have any conflict of interest.

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The well-studied canonical pathway is traditionally mediated by the inhibitor of κ B kinase complex (IKK α / β / γ) which leads to the phosphorylation and degradation of inhibitor of κ Bs (I κ Bs). Subsequently, RelA and p50 heterodimers are released to translocate into the nucleus to transcribe genes (Figure 1A and B). The less well-studied pathway, the non-canonical pathway, involves processing of p100 to p52 by NIK and IKK α . Both kinases phosphorylate p100, leading to the partial proteolysis of p100. The resulting protein p52 heterodimerizes with RelB to mediate the gene expression in the non-canonical arm of NF- κ B (Figure 1A and B). Various cytokines such as tumor necrosis factor α (TNF α) and interleukin-1 β (IL-1 β) activate the canonical pathway, resulting in an acute and transient response. The non-canonical pathway of NF- κ B can be activated by only a handful of ligands, such as CD40 ligand (CD40L), B-cell activating factor belonging to the TNF family (BAFF), ligands for the lymphotoxin β receptor (LT β R) and TNF-related weak inducer of apoptosis (TWEAK). The complexity of the regulation stems from the fact that these “non-canonical” pathway activators induce the canonical pathway as well.

NIK was first identified as a kinase that mediates the proximal signaling downstream of TNF and IL-1 receptors and the kinase activity of NIK is required for this process [1]. NIK also mediates stimulation through other receptors such as CD27, CD30, CD40, LT β R and BAFFR [2]. When overexpressed, NIK leads to activation of NF- κ B, therefore protecting cells from undergoing TNF-induced apoptosis [1]. In reverse, the kinase dead NIK mutant (either KK429/430AA or T559A) has a dominant negative effect and over-expression of this mutant inhibits activation of NF- κ B by TNF α [1,3]. Surprisingly, dominant negative NIK has a differential effect on NF- κ B activation in transformed cell lines versus primary cells. Adenoviral expression of dominant negative NIK in primary macrophages did not have an inhibitory effect on NF- κ B activation in response to TNF α and lipopolysaccharide (LPS), as suggested by normal NF- κ B dependent cytokine production, I κ B α degradation and NF- κ B DNA [4]. Although it is not clear why dominant negative NIK exerts different effects on different cell types, NIK was thought to be an essential kinase for the canonical NF- κ B activity.

NIK has been demonstrated to interact with and activate IKK α and IKK β . However, NIK phosphorylates IKK α to a greater extent than IKK β [5]. Thus, NIK serves as an upstream IKK complex kinase responsible for the proximal signaling of various cytokine receptors. Since the identification of NIK, many other kinases have been identified as IKK kinases acting in concert with NIK. Some of them include MAP/ERK kinase kinase 1 (MEKK1) [6,7] and TGF- β activated kinase 1 (TAK1) [8]. TAK1 was identified as an upstream kinase of NIK that can activate NIK/IKK/NF- κ B signaling downstream of IL-1R [9]. TAK1 phosphorylates NIK at a site other than Ser-549, Thr-552 and Thr-559 since mutations of these amino acids to alanine still gave rise to phosphorylated NIK [9]. Although one study demonstrated that TAK1 did not directly phosphorylate IKK α [9], another study suggested that TAK1 may directly activate NF- κ B, independent of NIK [8]. NIK is also downstream of TNF receptor associated factor (TRAF) 2, 5 and 6 and TANK-binding kinase-1 (TBK-1) in NF- κ B activation upon certain ligand stimulation [2,10]. Additional proteins have been identified to activate NIK. Recently, it was reported that B-cell CLL/lymphoma 10 (Bcl10) phosphorylates NIK in a human colonic epithelial cell line treated with an inflammatory agent named sulfated polysaccharide carrageenan (CGN) [11].

IKK α is a preferential substrate for NIK. NIK directly phosphorylates IKK α on Ser-176 and mutation of this residue to alanine blocks NIK activation of IKK α , thus disrupting signals from IL-1 and TNF α stimuli [12]. Interestingly, NIK still phosphorylates the catalytically inactive IKK α (K44M), but does not induce phosphorylation of catalytically inactive IKK β (K44A), suggesting that autophosphorylation is necessary for IKK β response to NIK [13]. T-loop mutants of IKK α and IKK β (SS176/180AA and SS177/181AA, respectively) show

similar results. Mutations in the T-loop of IKK β disrupts NIK-induced phosphorylation, and the kinase activation status [13]. Nevertheless, T-loop mutations in IKK α do not affect NIK's ability to phosphorylate IKK α [13]. This suggests that NIK is a better kinase for IKK α than for IKK β [13]. Alternatively, the catalytic activity of IKK β may be required to "prime" NIK phosphorylation of IKK β . However, IKK β is considered a more potent activator of NF- κ B since co-expression of NIK and IKK α does not activate NF- κ B to the same degree over-expression of IKK β alone [5].

In contrast to the report by Delhase *et al.* [13], O'Mahony *et al.* demonstrated that NIK can stimulate phosphorylation of IKK β (K44A) in an IKK α dependent manner [14]. In fact, IKK α appears to be upstream of IKK β in response to NIK. The IKK α (S176A) mutant is unable to phosphorylate IKK β in response to NIK activation [14]. Thus, this study suggested a directional activation of IKK α towards IKK β , stimulated by upstream kinases such as NIK [14]. Remarkably, NIK recruitment by IKK α to the IKK complex was thought to be required for the basal activation of IKK β [14], despite the non-essential role of NIK in response to certain stimuli. Additional insights on NIK regulation of the IKK complex result from studying the IKK-complex-associated-protein (IKAP) scaffold protein. IKAP forms a complex with IKK α , IKK β and NIK, and has a higher affinity for NIK compared to IKK α and IKK β [15]. In a kinase assay from HEK293 over-expressing IKK α and/or, IKK β and/or, NIK, inactive NIK completely inhibits the kinase activity of the IKK complex, suggesting that NIK has a tight regulation on the IKK kinase activity [15].

From the preferential interaction with IKK α , it can be deduced that NIK is a more important kinase for IKK α than IKK β . Later studies suggest that NIK is only essential for the non-canonical pathway, although it plays a role in both the canonical and non-canonical pathways of NF- κ B activation. Data from studies using mouse embryonic fibroblasts (MEFs) with NIK knock-out or with a naturally occurring NIK defective mutant, alymphoplasia (*aly/aly*), support this notion. MEFs from these mice exhibit an intact response to TNF α , yet a partial defect in response to LT β R ligands [16,17]. In addition to these mouse models, expression of dominant negative NIK in human skin fibroblasts only inhibits lymphotoxin α 1 β 2 (LT α 1 β 2)-induced NF- κ B signaling, but does not affect TNF α -induction [4]. More careful characterization suggests that in NIK^{-/-} MEFs, NF- κ B still binds to DNA yet mRNA of some NF- κ B responsive genes such as I κ B α and monocyte chemotactic protein-1 (MCP-1)/CCL2 are not induced in response to LT β R agonist antibody [16]. Thus, it was speculated that NIK does not affect I κ B degradation and nuclear translocation but NIK can activate the transcription of NF- κ B regulated genes. These data are consistent with the findings that RelA and p50 binding to the DNA is normal in *aly/aly* mice [18]. In *aly/aly* mice, the situation may be more complex. In response to LT β R ligation, the vascular cell adhesion molecule-1 (Vcam-1) protein expression level remain unchanged in *aly/aly* cells, assessed by flow cytometry analysis [19,20]. However, LT β R ligation still increases mRNA expression of Vcam-1 by inducing IKK kinase activity in *aly/aly* MEFs [20]. These data suggest that *aly* mutation does not affect the canonical pathway induction via LT β R, but may affect the post-transcriptional regulation of Vcam-1 [20].

Notably, NIK deficiency affects activation of both the canonical and non-canonical pathways in response to certain ligands in specific cell types. For instance, NIK deficient lymphocytes are defective in activation of the canonical and non-canonical pathways of NF- κ B upon treatment with CD40L, BAFF and CD70 (a ligand for CD27) [21]. Deficiency of NIK results in defective I κ B degradation, RelA nuclear translocation, and p100 to p52 processing in response to these ligands [21]. Intriguingly, NIK deficiency in B-cells results in slight diminution of some NF- κ B members such as p100, RelB and c-Rel, all of which are NF- κ B-regulated genes [21]. This observation implies that continuous and weak basal NF- κ B activation exists in a NIK-dependent manner [21]. B-cells from *aly/aly* mice also exhibit

diminished I κ B phosphorylation in response to CD40 engagement, whereas dendritic cells do not have a similar defect [22]. Thus, the notion that NIK is non-essential for the canonical pathway is somewhat misleading. Signals via CD40 and BAFFR, in fact, rely on NIK to induce RelA activation. In other words, NIK is not required for the proximal signaling from the receptors inducing only the canonical pathway, yet it is required for the signaling from some receptors that activate both the canonical and non-canonical arms of NF- κ B. Ramakrishnan *et al.* proposed NIK-dependent and NIK-independent mechanisms of receptor activation. Based on their data, they suggested that NIK is recruited together with the IKK complex only to the receptors that are NIK-dependent (i.e. CD27), whereas it does not associate with the receptors that are NIK-independent (i.e. TNFR) [21]. The necessity for NIK in the canonical NF- κ B pathway may be cell type and ligand specific and how this specificity occurs is yet to be determined.

The requirement of NIK for the non-canonical pathway is more evident. NIK is responsible for the processing of p100 to p52, confirming its role in the non-canonical pathway [23]. The kinase activity of NIK or IKK α is not required for the recruitment of p100 to both kinases however it is required for the ligand-induced proteolysis to occur [24]. Surprisingly, NIK is not required for the constitutive processing of p100 to p52 [25]. The mechanism by which NIK controls p100 to p52 processing is through its stability. Upon stimulation with ligands such as CD40L or BAFF, basally translated NIK is stabilized to induce p100 to p52 processing [26]. The C-terminus of NIK is crucial for the interaction with p100 as well as for inducing the proteolysis of p100 [23]. In addition to regulating p100 proteolysis, NIK plays a role in histone modification to control gene expression together with IKK α . In response to the bacteria endotoxin LPS, NIK induces phosphorylation of IKK α , which in turn phosphorylates histone H3 [27]. This is one mechanism by which NIK controls LPS-induced NF- κ B activation and gene expression.

From the above evidence, it is apparent that NIK is necessary for the non-canonical pathway. Paradoxically, NIK was first identified as a kinase which activates the canonical pathway downstream of TNF and IL-1 receptors. The physiological relevance of NIK in the canonical NF- κ B activation pathway was re-investigated by Zarnegar *et al.* who demonstrated the role of NIK in amplification of signals from the canonical pathway [28]. They used TRAF3^{-/-} mice to show the elevation of the canonical pathway activity as a result of TRAF3 deficiency and NIK stabilization [28]. In concert with findings from NIK over-expression studies, NIK activates the canonical pathway when present at a high amount due to stabilization. Interestingly, the response of MEFs to TNF α was markedly enhanced when pre-treated with LT β R agonist antibody [28]. This enhancement was not observed in NIK^{-/-} MEFs, suggesting that NIK stabilization in response to pre-treatment with LT β R agonist antibody amplified signals upon TNF α treatment [28] (Figure 1B). As the canonical and non-canonical pathways are traditionally thought to be inflammatory and lymphoid organogenic responses, respectively, it is possible that NIK may have a role in integrating signals from those processes.

In summary, NIK is a kinase that functions in the canonical pathway, but is required only for NF- κ B activation in response to certain ligands. The physiological significance of this selective requirement remains elusive. Possibly, NIK lies at a cross road of the canonical and the non-canonical pathway of NF- κ B, maintaining the positive and negative regulatory balance between these two pathways. For example, NIK activates the canonical pathway and thus possibly induces the production of p100, a fourth I κ B of the NF- κ B family [29] (negative feedback mechanism). At the same time, NIK is the kinase responsible for the processing of this I κ B so that it will liberate NF- κ B heterodimers such as RelA/p50 to activate gene transcription (positive feedback mechanism). Another significance of NIK's role in both pathways is the amplification of signals as described above [28]. It becomes

exceedingly clear that NIK plays a dynamic role in NF- κ B activation, coordinating the intricate balance between the canonical and non-canonical regulations of NF- κ B. Maintaining this balance is crucial for the proper physiological responses. Moreover, tipping this balance probably leads to disease processes such as cancer.

1.2. Chemokines, cytokines and genes under the influence of NIK

NIK influences the production of inflammatory cytokines and chemokines, possibly by regulating the non-canonical pathway, or in some contexts, by regulating both the canonical and non-canonical pathways. It is noted that inflammatory factors such as interferon-inducible protein-10 (IP10)/CXCL10, MCP-1, KC and regulated upon activation, normally T-cell expressed, and presumably secreted (RANTES)/CCL5 are upregulated in TRAF2^{-/-} mice and more so in TRAF3^{-/-} mice, as these knock-out have a dramatic stabilization of NIK protein expression [30,31]. Similarly, the IL-12 level is elevated in TRAF3 deficient tissues [28]. However, the contribution of the canonical or the non-canonical pathway in up-regulating these secreted factors is not clear, since NIK significantly activates the canonical NF- κ B pathway when it accumulates as a result of the loss of TRAF3 (TRAF3^{-/-} mice) [28]. T-lymphocytes from *aly/aly* mice fail to secrete IL-2, suggesting that NIK regulates IL-2 production [32]. Additionally, Sanchez-Valdepenas *et al.* have shown that NIK regulates the phosphorylation of c-Rel, the binding of c-Rel to a regulatory region within IL-2 promoter [CD28-responsive enhancer (CD28RE)], and controls IL-2 transcription in T-lymphocytes [33]. Thus, NIK may influence cytokine expression in an IKK/I κ B/NF- κ B pathway independent manner. Transcription of other cytokines and chemokines such as IL-6 and IL-8/CXCL-8 are also likely to be NIK-dependent as kinase-dead NIK inhibits their production upon LT β R ligation [4].

Other genes regulated by NIK include TRAF2 and TRAF3, both of which are negative regulators of NIK [34,35]. Moreover, NIK appears to positively regulate its own transcription [36]. Microarray data from a colon carcinoma cell line with TRAF3 depletion by siRNA show that the NIK mRNA level is elevated in these cells [36]. These data suggest that stabilization of NIK protein through loss of TRAF3 leads to a positive feedback loop, resulting in enhanced transcription of NIK. Although the universality of this effect remains to be elucidated, regulation of NIK appears to have built-in positive and negative feedback systems, implying that intricate control is necessary for the appropriate physiological responses.

Intriguingly, NIK affects the expression of members of the NF- κ B pathway. RelB, p50, and c-Rel expression levels are reduced in *aly/aly* mice [32] as well as in NIK knock-out cells [21]. Although the list of genes regulated by NIK described here is not exhaustive, we can conclude that NIK modulates chemokines, cytokines and signaling molecules in NF- κ B pathways, implying that NIK can have a profound effect on NF- κ B activation.

2. Regulation of NIK

2.1. Understanding the molecular components of NIK

NIK was first identified as a mitogen activated kinase kinase kinase (MAP3K) that binds to TRAF2 in a two-hybrid screen of a human B-cell cDNA library [1] and was later shown to phosphorylate IKK α and IKK β . In the two-hybrid screen, NIK did not bind to TNF receptor associated death domain protein (TRADD), Fas-associating protein with death domain (FADD) or receptor-interacting protein-1 (RIP-1) [1]. NIK shares some homology to the MAP3K. From this homology and from mutagenesis studies, it was determined that Thr-559 in the activation loop of NIK is required for its kinase activity [3]. Unlike RIP-1 whose activity is not required for NF- κ B activation, the kinase activity of NIK is necessary for both

basal and TNF α -mediated NF- κ B activations [1,3] as well as for the processing of p100 to p52 [23]. Curiously, NIK can spontaneously homodimerize or oligomerize, but TNF α does not further induce oligomerization of NIK [3]. Thus, oligomerization or dimerization of NIK may be crucial only for the basal, but not for the ligand-induced kinase activity of NIK. Another layer of regulation of NIK may come from the intramolecular interaction of NIK. The amino-terminal (N-terminal) region of NIK contains a negative regulatory domain, which interacts with the NIK carboxyl-terminal (C-terminal) end. This interaction prevents NIK from binding to its substrates, such as IKK α [37].

Mutant forms of NIK have been generated to provide insight into the functional domains of NIK, and to determine how regulation of NIK is achieved. Based on its sequence and functions, at least four distinct domains were identified in NIK: the basic region, the proline rich region, the kinase domain and the C-terminal TRAF binding domain [37] (Figure 2). Among these, the C-terminus is responsible for many important interactions. The C-terminus binds to IKK α , the downstream kinase of NIK, and also mediates homotypic dimerization or oligomerization [3]. A careful characterization of NIK demonstrated that the C-terminal fragment of NIK (aa 735–945) is necessary and sufficient for binding to IKK α [3]. Not surprisingly, expression of the C-terminus of NIK disrupts the activation of NF- κ B by TNF α as well as by the wild type NIK over-expression [1,3]. In parallel, the kinase dead NIK mutant acts as a dominant negative mutant when over-expressed [1,3], possibly due to its ability to bind to IKKs, while lacking the kinase activity. The kinase dead NIK (KK429/430AA) also cannot process p100 to p52 [23], suggesting that NIK kinase activity is required for the non-canonical NF- κ B pathway.

The TRAF binding domain of NIK, as the name suggests, mediates the binding of NIK to TRAFs such as TRAF1, 2 and 6 [1]. Unlike other TRAFs, TRAF3 binds NIK in the N-terminal region of NIK (aa78–84). Deletion of this domain results in the dramatic stabilization of NIK since TRAF3 mediates its degradation [35,38]. In addition to domains that regulate NIK stability, the protein also has nuclear import and export signals at the N-terminus (aa143–149) and the C-terminus (aa770–779/aa795–805), respectively [39]. These signals enable the nucleo-cytoplasmic shuttling of NIK. A truncation mutant of NIK which lacks the nuclear export signal, thereby confining NIK to in the nucleus, still retains some ability to activate NF- κ B [39]. These data suggest that NIK may be able to activate the nuclear IKK complex, or it may have a direct effect on the transcription mediated by NF- κ B. NIK also appears to localize to the nucleolus, as determined by the signal RKKRKKK (aa143–149) from the protein's N-terminus [40]. This short stretch of amino acid sequence is both necessary and sufficient for the nucleolar localization of NIK [40]. Deleting the nucleolar localization motif attenuates NIK's ability to activate NF- κ B [40], suggesting that NIK may have additional roles in different cellular compartments, aside from acting as an IKK kinase.

In addition to genetically engineered mutants, there exists a naturally occurring mutation of NIK in mice named alymphoplasia or *aly* mutation, first described by Miyawaki *et al.* in C57BL/6J mice [41]. Shinkura *et al.* later mapped the gene responsible for this mutation and identified a missense point mutation (G855R) in the carboxyl terminus of NIK, which is necessary for TRAF binding [17] (Figure 2). Despite the speculation that *aly* NIK may not bind to one of the TRAFs, co-immunoprecipitation experiments suggest otherwise. At least the C-terminus of NIK (aa624–947) (TRAF-interacting domain) carrying the *aly* mutation binds to TRAF 1, 2, 3 and 6 as equally as the wild type C-terminal fragment binds [42]. Yet, the C-terminus of *aly* NIK does not bind IKK α and binds IKK β with less efficiency compared to the wild type C-terminus [42], implying that phenotypes of *aly* stem from the defective NIK/IKK α interaction.

Interestingly, when over-expressed in 293T cells, *aly* NIK can still activate NF- κ B, as manifested by an increased DNA binding of NF- κ B in electrophoretic mobility shift assay (EMSA) [17]. Since *aly* NIK fails to bind IKK α , perhaps with its over-expression there is sufficient NIK interaction with IKK β to activate NF- κ B. The ability of *aly* NIK to activate NF- κ B is in contrast to the kinase dead NIK, which fails to activate NF- κ B. Perplexing views come from the fact that *aly* NIK inhibits CD40-mediated NF- κ B activation in B-cells [42]. Likewise, this mutant form of NIK inhibits NF- κ B activation, caused by TNFR1 and 2 over-expression but not LT β R-mediated NF- κ B activation in 293T cells [17]. The studies on the ability of the *aly* NIK to activate or disrupt NF- κ B did not distinguish clearly the contribution of NIK to the canonical or non-canonical pathway, as the conclusions were based on EMSA or luciferase activity. Moreover, how the *aly* mutation results in a dominant negative function of NIK in response to certain ligands remains elusive. Yet, all these data suggest that the *aly* mutation may disrupt NF- κ B activation in a ligand-specific manner.

Additional studies addressed the role of the *aly* mutation in the non-canonical NF- κ B pathway. The *aly* mutation disables NIK's ability to bind either IKK α or p100, resulting in a deficient p100 to p52 processing [23,42]. MEFs from *aly/aly* mice are defective in inducing mRNA level of Vcam-1 expression upon ligation with LT β R agonist antibody [17]. These data are reminiscent of data from NIK knock-out mice, confirming the notion that NIK is indispensable for the non-canonical pathway of NF- κ B. *Aly* mutation highlights the importance of the physical interaction between NIK and its substrate. Despite the presence of the kinase domain, NIK function may still be defective when the proper binding does not occur. It is very likely that the physical interaction between its substrates as well as the kinase activity is required for the ability of NIK to stimulate p100 to p52 processing. In other words, those two criteria are required for the non-canonical pathway, whereas the interaction between NIK and its binding partner at the C-terminus may not be absolutely necessary for NIK activation of the canonical pathway of NF- κ B.

2.2. Regulation of NIK by the stability and dosage

Although NIK functions as a kinase and phosphorylation of NIK is required for its ability to activate IKKs and NF- κ B, regulation of the NIK protein stability is the major mechanism by which NIK function is regulated. Interestingly, mRNA expression of NIK is increased in the absence of its 3' untranslated region, suggesting that NIK expression may be mediated at the post-transcriptional level [1]. The basal level of NIK protein is very low under normal physiological conditions but NIK is stabilized and its half-life is increased to about three hours after stimulation with CD40L and BAFF in B-cells [26]. The basal stability of NIK depends on the action of four E3 ubiquitin ligases namely; TRAF2, TRAF3, cellular inhibitors of apoptosis 1 and 2 (cIAP1 and cIAP2). Initially, TRAF3 was suggested to degrade NIK, since the direct interaction between NIK and TRAF3 suppressed NIK activation of NF- κ B [38]. Deletion of the TRAF3 binding domain (aa78–84) in NIK prevents NIK from being ubiquitinated and degraded [38]. Consistently, in a knock-in mouse with NIK mutant lacking the TRAF3 binding domain, NIK was substantially stabilized [35]. Additional studies suggest that TRAF3 negatively regulates NIK. TRAF3 is downregulated in response to CD40L and BAFF [43,44]. Interestingly, CD40L and BAFF treatment protects basally translated NIK from undergoing proteasomal degradation in B-cells [26]. Since both CD40L and BAFF activate the non-canonical pathway of NF- κ B, and NIK is an essential kinase for this pathway, it can be deduced that downregulation of TRAF3 upon receptor ligation leads to the positive regulation of NIK, relaying downstream signals. In consistent with the previous data, TRAF3^{-/-} mice have constitutive p100 processing due to the accumulation of NIK [45].

In addition to TRAF3, TRAF2 is known to play a negative role in p100 to p52 processing, as TRAF2^{-/-} B-cells have the constitutive processing of p100 [46]. Similarly, cIAP1 and 2 are

implicated in the negative regulation of NIK by studies using IAP inhibitors. Pharmacological inhibitors of IAPs, which lead to auto-degradation of cIAP1 and 2, markedly stabilize NIK and stimulate the activation of NF- κ B through the non-canonical pathway [47]. These data strongly suggest that TRAFs and cIAPs are degradative agents of NIK.

To further explore the negative regulatory complex of NIK, two studies provide biochemical evidence of how TRAF2, TRAF3, cIAP1 and cIAP2 cooperate to affect the stability of NIK. According to data from Vallabhapurapu *et al.* and Zarnegar *et al.*, TRAF2 preferentially binds to cIAP1/2 and TRAF3 preferentially binds to NIK. TRAF2 is required to recruit the TRAF3/NIK complex to the TRAF2/cIAP1/2 complex and TRAF3 is required to recruit the TRAF2/cIAP1/2 complex to TRAF3/NIK. Based on their data with knock-out MEFs, TRAF2 and TRAF3 cannot compensate for each other's loss in degrading NIK [30,31]. On the contrary, cIAP1 and cIAP2 can compensate for the loss of one another in degrading NIK and downstream processing of p100 [31]. To add to the complexity of the system, NIK also regulates TRAF2 and TRAF3 expression at a transcriptional level [34,35]. When NIK is stabilized in B-cells, it induces mRNA expression of TRAF2 and TRAF3, establishing a negative feedback loop for NIK [35]. This negative feedback regulation is likely to be the mechanism to ensure the termination of NF- κ B signaling after an appropriate duration.

In slight contrast to the requirement of TRAF3 to mediate NIK degradation by recruiting the TRAF2/cIAPs complex, Demchenko *et al.* have shown in the multiple myeloma model that TRAF2 is still capable of binding to NIK and inducing degradation even when TRAF3 interaction with NIK is absent [48]. The investigators of this study argued that TRAF3 enhances the degradation of NIK, yet it is not absolutely required for this process to occur [48]. The different results regarding the requirement for TRAF3 may be due to the transformation status of the B-cell-derived multiple myeloma cells. Perhaps not all of the four E3 ubiquitin ligases are functionally equal in all cell types, some being more important for ablation of NIK than others.

The reason for the necessity of four ubiquitin ligases for the constitutive degradation of NIK is not known. Although the field has made a significant progress in elucidating the regulation of NIK, many questions remain unanswered. Among four ubiquitin ligases that are responsible for NIK degradation, which is/are the actual ubiquitin ligase(s) that conjugate ubiquitin to NIK? Liao *et al.* suggested that using purified TRAF3 in an *in vitro* ubiquitination assay did not result in significant ubiquitination of NIK [38]. Consistent with this finding, Vallabhapurapu *et al.* argued that TRAF3 was simply an adaptor, as they did not observe a necessity for the TRAF3 RING domain to mediate NIK degradation [30]. Instead, they proposed that cIAP1/2 may be responsible for K-48 ubiquitination of NIK [30]. In support of this idea, it is demonstrated that RING mutant constructs of TRAF2 or cIAP1 do not degrade NIK [30,47]. Hence, only some of these E3 ubiquitin ligases may serve as the critical ubiquitin ligase for NIK degradation, whereas others merely serve as scaffold proteins. Perhaps, it is quite challenging to reconstitute an *in vitro* ubiquitination of NIK, possibly due to the requirement of all these factors and the ubiquitination activity that they have on one another. In addition, we still do not know the identity of the lysine residue(s) on NIK required for its ubiquitination.

In addition to the basal regulation of NIK, some mechanisms by which NIK is stabilized upon ligation of upstream receptors have been demonstrated (Figure 1B). It is noted that upon ligand stimulation by CD40L or BAFF, TRAF2 and TRAF3 are degraded in a cIAP-dependent manner, resulting in the stabilization of NIK [30, 31]. TRAF3 degradation precedes TRAF2 degradation, suggesting that TRAF3 degradation first disassembles the complex bound to NIK to relieve the suppression [30, 31]. The mechanism of how cIAPs

become the degradative agents for TRAFs upon ligand engagement remains elusive. Another ligand, TWEAK, mediates NIK stabilization in a manner slightly different from CD40L and BAFF, at least in the melanoma cell line A2058. Upon TWEAK ligation, cIAP1 is degraded and stabilized NIK potentiates p100 to p52 processing [47]. However, cIAP-dependent negative regulation of NIK may not be the complete story. A recent report suggests that cIAP-independent NIK stabilization may exist, as TWEAK can still stabilize NIK in cIAP1/2 double knock-out mice [49].

Another receptor that can activate NIK is the LT β R. It was recently proposed that both LT β R and NIK bind to the same site on TRAF2 and 3. Thus, upon stimulation through LT β R, the receptor competes for the binding sites on TRAF3, thus replacing NIK from the negative regulatory complex and relieving the degradation of NIK [50]. Similarly, the receptor replaces cIAP1/2 from TRAF2, possibly to ensure the disassembly of the degradative complex for NIK [50]. Aside from serving as an allosteric competitor, activation through LT β R leads to ubiquitination and proteosomal degradation of TRAF3 in a TRAF2-dependent manner [50]. Although TRAF2 is also ubiquitinated and degraded upon ligand stimulation via LT β R, it is not clear whether TRAF2 undergoes auto-ubiquitination or whether some other E3 ligases are responsible for this process. Interestingly, cIAPs were not associated with the activated LT β R and TRAF complex [50]. Thus, the mechanism by which NIK is stabilized upon ligand induction appears to be unique for individual receptor signaling.

Another interesting question about NIK regulation is whether it has a dosage dependent effect on NF- κ B activation. The first indication that the level of NIK may be tightly regulated comes from NIK over-expression and gene knock-out studies. NIK potentially activates basal as well as ligand stimulated NF- κ B upon over-expression [1]. However, data from NIK knock-out mice suggest that this kinase is not required for the activation of the canonical pathway. Thus, the level of NIK likely determines whether NIK is selectively activating one or both pathways of NF- κ B. For example, in TRAF3^{-/-} mice where NIK is stabilized, nuclear accumulation of RelA, p50, RelB and p52 is significantly elevated, suggesting that there is a constitutive activity of both canonical and non-canonical pathways of NF- κ B [28]. Remarkably, the lethality resulting from TRAF3 deletion was significantly rescued by deleting just one allele of NIK [31]. This observation suggests that the level of NIK is tightly controlled to regulate physiological processes.

In accordance with the dosage dependent regulation of NIK, Demchenko *et al.* proposed a model by which the level of NIK protein may be tightly controlled [48]. In this model, TRAF2, TRAF3, cIAP1 and cIAP2 cooperatively modulate the stability of NIK. Only in the functional presence of all four of them, can maximal degradation of NIK be achieved. When one of the E3 ligases is absent or non-functional, NIK stability increases, resulting in NF- κ B activation. These investigators also postulated that initial stabilization of NIK robustly activates NF- κ B, whereas additional stabilization of NIK has minimal additive effect of NF- κ B activation [48]. The differential effect of NIK on NF- κ B activation depending on the dosage may help explain why the expression level of NIK is maintained minimal under normal physiological conditions.

In summary, there are three ways to regulate the function of NIK based on the literature up to date; phosphorylation, interaction between NIK and its binding partners, and the stabilization of NIK. The resulting NIK activity is the net effect of the interplay among these regulations.

3. NIK interacting proteins and the implication of these interactions

Yeast-two-hybrid and mass spectrometry analyses have identified expected as well as novel NIK interacting proteins. There are also other identified NIK regulating proteins that are not visible in mass spectrometry or yeast-two-hybrid analyses. Many of these NIK interacting proteins were identified due to their ability to modulate NF- κ B activity. Some NIK protein interactions, namely IKK α / β and p100, are expected based upon our knowledge that NIK functions to regulate NF- κ B signaling. However, other NIK protein/protein interactions, such as interaction with Hsp90, are somewhat unexpected. Although some of these proteins have been known to interact with the NF- κ B pathway, their functions in relation to NIK have not been fully explored. Exploration into the nature of these interactions may reveal novel functions for NIK and may uncover key information on the modulation of NIK protein.

NIK interacts with IKK α and IKK β (details discussed in “NIK in the regulation of NF- κ B”) in addition to its negative regulators, cIAP1, cIAP2, TRAF2 and TRAF3 (details discussed in “Regulation of NIK by the stability and dosage”). In addition to TRAF2 and 3, NIK associates with other TRAF family members, TRAF1, 5 and 6, and those interactions have been implicated in TNF and IL-1 induced NF- κ B activation [51]. It was also demonstrated that IKK γ can bind to NIK [52,53]. These interactions modulate NF- κ B activation in response to different stimuli.

Beside the E3 ubiquitin ligases, there are additional NIK interacting proteins that regulate the stability of NIK. NIK binds to another negative regulator called Monarch-1, which then induces the proteosomal degradation of NIK [54]. Another NIK interacting protein, Hsp90, also affects NIK stability by protecting NIK from autophagic degradation [55]. NIK physically interacts with Hsp90 through its C-terminal domain [55], although whether this interaction is direct or indirect is yet to be examined. Treatment of cells with the Hsp90 inhibitor geldanamycin dissociates NIK and Hsp90, and results in marked NIK degradation [55]. Thus, Hsp90 appears to have a positive regulation on the stability of NIK. On the contrary, another report demonstrated that Hsp90 is required for the negative regulatory role of Monarch-1 to induce NIK degradation [54]. Thus, depending on the context, Hsp90 can positively or negatively regulate the stability of NIK. By modulating NIK stability, these proteins regulate p100 to p52 processing and possibly downstream gene expression. From the literature, we can deduce that many layers of degradative mechanisms may be in place to maintain a physiologically appropriate level of NIK.

In addition to destabilizing interactions, NIK associates with one negative regulator that does not affect its stability. A yeast-two-hybrid screen of a human brain cDNA library using the N-terminus of NIK identified a novel NIK interacting protein named TRAFs and NIK-associating protein (TNAP). A GST-pull down assay using bacterially purified and *in vitro* translated NIK and TNAP proteins reveal that TNAP directly interacts with NIK [56]. TNAP can markedly decrease basal, TNF α -induced and NIK over-expression-mediated NF- κ B activation [56]. TNAP attenuates the kinase activity of NIK, as evidenced by reduced phosphorylation of the NIK substrate, IKK α [56], yet it does not appear to affect NIK stability. Furthermore, TNAP negatively regulates p100/p52 processing as well as NIK-mediated RelA phosphorylation, suggesting that it inhibits both canonical and non-canonical NIK activities [56]. Another protein identified from this yeast-two-hybrid screen is NIK and IKK β binding protein (NBIP). NIK directly binds to NBIP along with IKK β , and NBIP potentiates NF- κ B activation induced by these two kinases upon cytokine treatment [57]. Similar to TNAP, Raf kinase inhibitor protein (RKIP) antagonizes NIK in its ability to activate NF- κ B by interacting with the kinase [58]. In addition to NIK, RKIP inhibits the ability of TAK1, another IKK kinase, thus hampering NF- κ B activation [58]. Yet, RKIP

may not directly associate with these kinases, based upon negative data from the *in vitro* pull down assay using recombinant proteins [58].

Intriguingly, NIK interacts with death effector domain (DED)-containing proteins such as caspases 8, 10 and cellular FLICE like inhibitory protein (cFLIP) and these proteins activate NF- κ B, possibly through their interaction with NIK and IKKs [59]. Over-expression of dominant negative NIK disrupts the activation of NF- κ B, induced by these proteins [59]. Similarly, NIK also associates with the viral DEDs-containing protein vFLIP from human herpesvirus 8 (HHV8), which has an ability to activate NF- κ B [60]. These results suggest that NIK is possibly involved in the signaling from death receptors, determining survival or cell death through NF- κ B activation. Nevertheless, the significance for caspase 8 and its homologs in induction of NF- κ B through activation of IKKs and NIK remains to be elucidated.

Interestingly, epidermal growth factor receptor (EGFR) binds to NIK and RIP-1, forming a signaling complex to induce NF- κ B activation [61]. Over-expression of a dominant negative NIK disrupts EGFR-induced NF- κ B [61]. Thus, NIK may mediate cellular processes through tyrosine kinase receptor signaling. Consistently, screening NIK associating proteins by using antibody arrays has identified new binding partners of NIK that are also part of the EGFR complex [62]. FlagNIK expressing HEK293 was used to incubate with the antibody array and novel NIK binding partners were identified in the screen. In an over-expression system, NIK binds to growth factor receptor bound (Grb) family members such as Grb7, Grb10 and Grb14 [62]. Endogenous NIK also interacts with Grb7 and estrogen receptor 2 (Erb2), suggesting that NIK is part of the EGFR complex [62]. Experiments using NIK knock-out MEFs strongly support that NIK is required for EGFR activation of NF- κ B [62]. As EGFR is over-expressed in many breast cancer types, NIK may have a regulatory role in breast cancer progression.

Moreover, another activator of NIK, cancer Osaka thyroid-1 (Cot-1/Tpl-2) physically associates with NIK and IKK α [63]. Cot-1 induces phosphorylation of NIK, leading to the activation of IKK α [63]. Kinase deficient NIK disrupts Cot-1-induced activation of IKK α and subsequent NF- κ B activation in CD3/CD28 stimulation pathway [63].

Mass-spectrometry analysis of NIK associating proteins has been performed in the tandem affinity purification (TAP) tag system [53]. Some interesting proteins that were identified in this analysis include ribosomal proteins, DEAD-box proteins (DDX) and Cdc37 [for complete list, see [53]]. The functional significance of these interactions has not been fully explored. Biochemical and functional studies are necessary to identify new roles of NIK in the context of its interacting partners.

4. Crosstalk with non-NF- κ B pathways

In addition to NF- κ B activation, some reports suggest that NIK can activate other signaling pathways. In response to lymphotoxin-related inducible ligand that competes for glycoprotein D binding to herpesvirus entry mediator on T-cells (LIGHT), an activator of LT β R, NIK phosphorylates Stat3 on Tyr-705 and 727 [64]. Likewise, upon ligation through CD27, a TNFR family member, NIK mediates NF- κ B as well as stress-activated protein kinase (SAPK)/c-jun N-terminal kinase (JNK) activation, downstream of TRAF2 and TRAF5 [2].

In addition to TNF family members, interferons α/β (IFN α/β) can activate both the canonical and non-canonical pathways of NF- κ B [65]. Specifically, IFNs induce proteolysis of p100 to p52 in a NIK-dependent manner, generating p52/RelA dimers to activate NF- κ B regulated gene transcription [65]. Over-expression of dominant negative NIK inhibits IFN-responsive

NF- κ B transcription and protects cells from IFN-mediated cell death, implying that NIK plays a role in both processes [65]. Therefore, NIK may be involved in the crosstalk between IFN and NF- κ B signaling. Similarly, NIK can relay signals from a tyrosine kinase receptor, EGFR, to induce NF- κ B activity by associating with the EGFR complex [62]. These data suggest that diverse stimulus/receptor complexes can converge upon NIK to modulate downstream NF- κ B activity.

Aside from NF- κ B signaling, NIK appears to have a role in another inflammatory response, Nod-signaling. Nod1 and Nod2 recognize the bacterial product, muramyl dipeptide (MPD), to mount host responses. NIK binds to Nod2 and is required for MPD and LPS-induced p100 to p52 processing [66]. Furthermore, NIK^{-/-} bone marrow derived cells fail to induce B-lymphocyte chemoattractant (BLC)/CXCL13 transcripts when treated with MPD, LPS or a combination of both [66], suggesting that NIK regulates BLC gene expression in response to bacteria infection.

In agreement with the fact that NIK is a MAP3K, it also activates MEK1/2 and MAPK. NIK activates MEK1 and Erk1/2 in PC12 cells and regulates neurite process formation [67]. In addition, osteopontin (OPN) induces NIK phosphorylation and NIK downstream activity such as phosphorylation of IKK α/β and MEK1/Erk1/2, subsequently activating NF- κ B. Interestingly, OPN-induced NIK activation is $\alpha_v\beta_3$ -integrin-dependent [68], although the exact mechanism for this integrin involvement is not clear. Through the NIK/IKK complex and subsequent NF- κ B activation, OPN modulates matrix metalloproteinase-9 (MMP9)-dependent cell migration and invasion in melanoma cells [68].

5. The role of NIK in the regulation of the immune system

5.1. NIK in the regulation of B-cells

Based upon data accumulated from studies of the NIK null mice and the *aly/aly* mice, subsequent studies have often focused on NIK's role in the modulation of the immune system. *Aly/aly* mice were characterized as mice without lymph nodes and Peyer's patches [69]. These mice do not exhibit somatic hypermutation during antigen-specific immune response and germinal center formation [69]. Presumably because of these defects, *aly/aly* mice exhibited weak antibody response and defective isotype switching [69]. These mice also lack allogeneic skin rejection, exhibit abnormal spleen and thymic development and have defective B-cell proliferation [32,41,69]. Since NIK is a downstream activator of LT β R and LT β R is exclusively expressed by non-lymphoid cells, it was speculated that the phenotypes in *aly/aly* mice were caused by a defective signaling in non-bone marrow derived cells [19]. However, transferring bone marrow from *aly/aly* mice into heterozygous *aly* mice clearly shows that proper NIK activity is required intrinsically in bone marrow-derived cells as well as in stroma. Chimeric mice with bone marrow derived cells from *aly/aly* mice have poorly developed B-cell follicles in the spleen with lack of follicular dendritic cell clusters and germinal center formation [32]. B-cells from *aly/aly* mice also have a defect in proliferation and IgM/IgG production in response to CD40L, LPS and anti-Ig activation [22]. Interestingly, up-regulation of surface molecules such as MHC II and intercellular adhesion molecule-1 (ICAM-1) is not affected by *aly* mutation in B-cells [22].

In addition to the necessity of NIK activity in bone-marrow derived cells, it is also required in stromal cells in the peritoneal cavity so that B1 cells can emigrate. *Aly/aly* mice have defective B-cell emigration from the peritoneal cavity, resulting in accumulation of B-cells in the cavity [70]. These phenotypic defects are due to the inability of NIK deficient stromal cells to express Vcam-1 and ICAM-1, therefore weakening the interaction between the stromal cells and B-cells [70].

Most of the phenotypes in NIK deficient B-cells may be attributable to NIK's function as an activator of IKK α . However, intriguingly, NIK has other roles in addition to its role as an IKK α kinase in B-cells. To distinguish IKK α -dependent and IKK α -independent contribution of NIK to the development of B-cells, mice with IKK α phospho deficient mutant (S176A and S180A) were generated [71]. As these serine residues are phosphorylated by NIK, these mutations should mimic the functional absence of NIK. Results showed that *aly/aly* mice have impaired antibody production, yet IKK α (S176A/S180A) knock-in mice do not [71]. B-cells from these IKK α knock-in mice proliferate normally in response to natural ligands such as CD40L, BAFF or the bacterial product LPS, in contrast to the phenotypes in *aly/aly* mice [22,32]. Altogether, these data suggest that NIK may have IKK α and non-canonical NF- κ B pathway independent functions, that can profoundly affect the cellular physiology, at least in B-cells [71].

In addition to its role in B-cell function, NIK provides survival signals in B-cells. Under normal physiological conditions, signal transduction events activated through CD40 and BAFFR are necessary for the induction of p100 proteolysis and for the promotion of B-cell survival [72,73]. Consistent with this notion, ablation of NIK negative regulators, TRAF2 and TRAF3, specifically in B-cells markedly enhances the survival of B-cells [46,74,75]. Substantial NIK accumulation enables TRAF3^{-/-} B-cells to survive in a ligand-independent manner [45], as NIK stabilization bypasses the necessity of CD40L and BAFF engagement.

In support of the genetic studies, treatment of primary B-lymphocytes with secondary mitochondria-derived activator of caspase (SMAC) mimetic compounds increase the proliferation and survival of these cells [31]. SMAC mimetics degrade cIAPs, negative regulators of NIK, and therefore stabilize NIK expression. Although accumulation of NIK enhances the survival of B-cells, it impedes the transition from the pro-B to the pre-B cell stage, thus disrupting the normal development of B-cells [30]. NIK also has a suppressive role on the expression of the transcription factor Pax5, which regulates the pro-B to pre-B cell transition. The mRNA transcript level of Pax5 was reduced in Traf2^{-/-} and Traf3^{-/-} B-cells and was restored upon deletion of NIK in these cells [30]. Conversely, transgenic mice with TRAF3 specifically over-expressed in B-cells exhibited an expanded plasma cell population despite the low expression of NIK [76]. Yet, their proliferative response to stimuli such as CD40L and BAFF *in vitro* did not differ appreciatively from wild type cells [76]. Although it remains ambiguous how the level of NIK specifically affects the B-cell population, it is apparent that the NIK level has to be tightly regulated to give rise to a proper differentiation and expansion of B-cells.

5.2. NIK in the regulation of T-cells

During the development of T-cells, autoreactive T-cells have to be eliminated to establish self-tolerance. One way to achieve this is through the negative selection of T-cells as a result of their interaction with the thymic stroma where self-antigens are expressed. Generation of thymic chimeras using *aly/+* or *aly/aly* embryonic thymus suggests that NIK is required in stromal cells to prevent autoimmune diseases in some organs [77]. In addition, *aly/aly* mice have fewer CD4⁺CD25⁺ regulatory T-cells (T_{reg}) [77]. Reduction of this T-cell subset correlated with downregulation of forkhead box P3 (Foxp3) in CD4⁺ T-cells and down-regulation of autoimmune regulator in thymus, the former being a major transcription factor for T_{reg} and the latter being responsible for antigen production to establish self-tolerance [77].

Additional studies using NIK knock-out mice support the notion that NIK has a role in the T_{reg} population. T_{reg} cells have regulatory role to suppress auto-reactive effector T-cells. NIK knock-out mice have a significantly lower ratio of T_{reg} to total CD4⁺ T-cells, although their suppressive function is comparable to the wild-type mice [78]. Similar phenotypes

were observed using *aly/aly* mice [77]. In the absence of NIK, the T_{reg} population proliferates more upon stimulation through glucocorticoid-induced TNFR family-related gene (GITR), a TNFR family member [78]. Moreover, NIK knock-out mice have a lower subset of specific T_{reg} population, defined as CD62L^{high} CD4⁺CD25⁺ T cells, resulting in a higher proportion of CD62L^{low} CD4⁺CD25⁺ T cells. The physiological implication of this phenomenon is the impairment of the normal regulatory T-cell response, leading to autoimmunity [78].

In addition to its intrinsic role in T_{reg} maintenance, NIK also mediates self-tolerance by modulating the signals through CD40 and receptor activator of NF-κB (RANK) in medullary thymic epithelial cells (mTEC) [79]. During the development of T-cells, those reacting with the self-antigens are eliminated. mTECs are proposed to mediate this elimination of self-reactive T-cells by expressing self-antigens. Precursor mTECs culture from *aly/aly* mice did not exhibit RelB expression or nuclear localization when treated with CD40L or RANKL, thus these precursor cells did not fully develop into mTEC [79]. As NIK is downstream of CD40 or RANK signaling, NIK function is also necessary for the development of these cells [79].

Another way NIK is linked to autoimmunity is through its regulation in the Th17 cell population. Using NIK knock-out mice, it was demonstrated that NIK is necessary for Th17 differentiation [80]. NIK knockout mice are resistant to a T-cell mediated autoimmune disease named experimental autoimmune encephalomyelitis (EAE) due to the intrinsic defect in naïve CD4⁺ T-cell population to differentiate into Th17 cells [80]. Intriguingly, along with T-cell receptor (TCR) stimulation, Th17 differentiation can be co-stimulated by a TNFR family member, herpes virus entry mediator (HVEM), a receptor for LIGHT. This signaling is impaired in NIK knock-out T-cells, implying that NIK controls Th17 cells via LIGHT ligation [80]. NIK deficiency does not affect T-cell proliferation, yet it attenuates Th17-regulatory genes, such as IL-17, IL-21, IL-22, IL-23R and RORγt [80]. Th17 differentiation process may not be through the p100 to p52 proteolysis although this cannot be excluded. Instead, NIK deficiency results in diminished Stat3 phosphorylation upon activation through TCR as well as IL-6R [80]. Thus, NIK is necessary for synergistic Stat3 activation by these two receptors. It has already been reported that Stat3 is crucial for Th17 differentiation [81], and thus NIK is likely to have an impact on Th17 differentiation via this transcription factor. In addition to its effect on T_{reg} and Th17 populations, a subset of immature natural killer T-cells (NKT cells), referred to as Vα14i NKT, requires NIK-mediated RelB activation for development [82]. Thus, NIK plays a pivotal role in various populations of T-cells, modulating their development or differentiation.

NIK not only plays a critical role in T-cell differentiation and development, but it also has an effect on T-cell activation. TCR/CD3-stimulated T-cells cannot produce IL-2 in *aly/aly* mice [32]. In normal T-cells, NIK phosphorylates c-Rel in the transactivation domain and activates its transcriptional activity at CD28RE to regulate IL-2 production upon CD28 ligation [33]. The signaling through TCR and CD28 are thought to cooperate for optimal activation of T cells and cytokine production [83]. NIK has a role in regulating those processes through both receptors. Moreover, NIK and protein kinase C (PKC) cooperate in T-cell activation, as suggested by the defective proliferation in NIK defective mature T-cells. The NF-κB response cannot be sustained in thymocytes from mice with *aly* mutation in response to CD3 conjugation [84]. In parallel, thymocytes from *aly/aly* mice have a defect in the proliferative response upon stimulation with anti-CD3 antibody [84]. Therefore, it is speculated that NIK regulates the function of T-cells by modulating NF-κB activation when T-cells respond to antigens and in mediating T-cell proliferation [84].

5.3. Other immune regulation of NIK

Interestingly, in T and B cells isolated from the peritoneal cavity, NIK is downstream of secondary lymphoid organ chemokine (SLOC)/CCL21 receptor in activating NF- κ B [85]. Although it is well established that NIK is downstream of some cytokine receptors such as BAFFR, CD40 and LT β R, it was not previously connected to a seven-transmembrane G protein-coupled chemokine receptor. These peritoneal cavity cells (T- and B-cells) from *aly/aly* mice exhibit a defective homing to the gut-associated lymphatic tissue *in vivo* [85]. This homing defect partly contributes to the impaired immunoglobulin production of B-lymphocytes as they fail to migrate to the proper niches to become plasma cells [85].

The contribution of NIK in dendritic cells (DCs) has been elucidated by a few studies using *aly/aly* mice. DCs with *aly* mutation still have intact DC activation in response to engagement through CD40 [22]. DCs from *aly/aly* mice do not exhibit any difference in cell surface molecules such as MHC I, MHC II, CD80/86, CD40, CD70, 41BB-L, and CD150 or upregulation of chemokine receptors such as CCR2 and CCR7 [86]. Nevertheless, they are intrinsically defective in cross-priming with CD8⁺ T cells [86]. This defect is a result of *aly* mutant DCs inability to process the whole antigen [86]. DCs with *aly* mutation also fail to induce many genes, some of which are implicated in the survival of DCs (cFLIP and serpinb9) and antigen processing (cathepsin E and Lamp1) [86].

In addition to studying the functionally defective NIK, introducing wild type NIK into DCs provides insight into the function of NIK in DCs. It has been proposed that induction of NF- κ B through over-expression of NIK in DCs can be an effective vaccine adjuvant [87]. When expressed in DCs, NIK induces cytokine and chemokine production (such as TNF α , IL-6, IL-12, IL-15 and IL-18, IL-8, macrophage inhibitory protein-1 (MIP-1 α/β)/CCL3, MCP-1 and MCP-3) [87]. More importantly, when over-expressed in DCs, NIK enhances the antigen presentation, Th-1 type immune response, and antigen specific cytotoxic T-cell response *in vivo* [87].

NIK not only participates in the regulation of the adaptive immunity, it also has a role in innate immune regulation. NIK stimulates the transcription of cyclooxygenase-2 (COX-2) by phosphorylating a transcription factor PU.1, which binds to the promoter region of COX2 [88]. When NIK expression was silenced by siRNA, less PU.1 was recruited to the COX-2 promoter. In parallel, COX-2 production was impaired in NIK siRNA treated macrophages in response to LPS [88]. NIK also has a role in the inflammasome by interacting with a nucleotide binding domain-leucine rich repeat (NLR) protein, Monarch-1 [54,89]. Upon CD40 ligation, Monarch-1 and NIK association increases, resulting in proteosomal degradation of NIK and suppression of p100 to p52 processing in monocytes [54].

6. The role of NIK in other physiological processes

6.1. The role of NIK in viral and bacteria infection

Some viruses and pathogens potentially activate NF- κ B by using viral proteins that mimic cellular molecules. For example, some proteins from pathogenic sources can converge upon NIK to regulate NF- κ B activity. For instance, Epstein-Barr virus latent membrane protein 1 (LMP1) mimics constitutively active TNFRs to activate NF- κ B. Luftig *et al.* have demonstrated that a dominant negative NIK fragment (aa624–947) inhibits NF- κ B activation induced by LMP-1 expression, suggesting that LMP-1 utilizes NIK to activate downstream NF- κ B components [42]. Additionally, LMP-1 induces p100 to p52 processing and this induction is NIK and IKK α dependent [90]. In addition to LMP-1, the viral oncoprotein Tio from herpes virus atelans, that can transform simian and human T-cells [91], prevents NIK from undergoing constitutive turnover in Jurkat T-cells [92]. StpC from *Herpesvirus saimiri*, is another viral protein that activates NIK/NF- κ B pathway and has a transforming ability

[93]. Similarly, respiratory syncytial virus (RSV) induces the generation of p52 from p100 and this induction is dependent on both retinoic acid-inducible gene I protein (RIG-I) and NIK/IKK α . When co-immunoprecipitated, NIK was in complex with RIG-I and its downstream effector mitochondrial antiviral signaling protein (MAVS) and the C-terminus of NIK is required for this association [94]. By processing p100 via the NIK/IKK α complex, RSV releases RelA from p100 inhibition to translocate into the nucleus [94]. These viral proteins frequently activate the NIK/NF- κ B pathway in host cells and tissues.

In addition to activating NF- κ B as a viral response, NIK has a protective role during viral infection. *Aly/aly* mice are highly susceptible to viral infection (such as vesicular stomatitis virus, vaccinia virus and lymphocytic choriomeningitis virus) as they failed to mount appropriate antibody response, resulting from defective T-and B-cell interaction [95]. These data suggest that the function of NIK is required to protect the host from some viral infections.

The function of NIK is also important for the activation of NF- κ B by some bacteria. Nontypeable *Hemophilus influenzae*, a gram-negative bacterium, induces NF- κ B activation via the NIK/IKK α /IKK β signaling complex [96]. This activation was disrupted when a dominant negative mutant TAK1 was over-expressed, implying that TAK1 acts upstream of NIK and the IKK complex in this pathogenic pathway [96].

6.2. The role of NIK in the bone microenvironment

Osteoclasts and osteoblasts reside in the bone marrow, maintaining the balance between bone resorption and bone formation, respectively. This balance is important since it affects the general bone structure as well as the bone maintenance associated with pathological conditions such as cancer metastasis and arthritis. Research relating NIK in osteoclastogenesis has been of interest, as RANKL differentiates monocyte and macrophage lineage progenitors to become osteoclasts. RANKL is one of the few known activators of the non-canonical pathway that plays a crucial role in the bone microenvironment. NIK deficiency deters the nuclear translocation of RelA and p52 in response to RANKL stimulation, due to the role of p100 as an I κ B in osteoclast precursors. In other words, the lack of p100 proteolysis as a result of NIK loss prevents osteoclast formation [97]. In this scenario, the loss of NIK affects both pathways of NF- κ B and the phenotypes observed cannot be clearly attributed to either the classical or the non-canonical pathway of NF- κ B.

To distinguish the relative contribution of the two pathways, Vaira *et al.* re-expressed RelA or RelB in NIK^{-/-} osteoclast precursors [98]. They observed that only the re-expression of RelB rescues the differentiation defect of the osteoclast precursors, suggesting that RelB is necessary for the expression of factors required for osteoclast differentiation [98]. NIK itself is not required for the differentiation of these cells. Crossing NIK^{-/-} and p100^{-/-} mice gave rise to mice with normal osteoclast differentiation [98]. This observation suggests that NIK stimulation of the proteolytic processing of p100, a negative regulator of osteoclast differentiation, is necessary for the osteoclastogenesis (Figure 3). Amazingly, TNF α induces TRAF3 stabilization in osteoclasts [99], possibly destabilizing NIK, diminishing p100 processing and promoting osteoclastogenesis [100]. Unlike osteoclastogenesis, tumor-induced osteolysis in a B16 melanoma model requires both NIK and RelB, evidenced by the inhibition of tumor-induced bone loss in RelB^{-/-} and NIK^{-/-} mice injected with B16 melanoma cells [98]. Therefore, the non-canonical pathway of NF- κ B may have a therapeutic value in bone metastatic cancers.

As NIK function is needed for osteoclast differentiation, its role has been studied in an inflammatory arthritis model. Bone degradation in arthritis is performed by osteoclasts. Inhibiting the inducer of osteoclastogenesis, RANKL, ameliorates bone erosion in certain

arthritis models [101,102]. In serum transfer arthritis model, NIK is not required for inflammation, but for bone erosion [103]. By using B-and T-cell transfer into RAG2^{-/-} mice, Aya *et al.* elegantly demonstrated that NIK is necessary in T-cells, but not in-B cells, for antigen-induced arthritis [103].

Interestingly, NIK determines the fate of bone marrow stem cells through a peroxisome proliferator-activated receptor γ (PPAR γ)-dependent mechanism. Gene targeting of PPAR γ in mice revealed that PPAR γ induces adipogenesis [104]. Cytokines such as TNF α and IL-1 can prevent mesenchymal stem cells from differentiating into adipocytes. The adipogenesis suppression mediated by these cytokines is through activation of TAK-1 binding protein (TAB)/TAK1/NIK signaling [105]. Over-expression of NIK in ST2 cells, a mesenchymal cell line, diminishes PPAR γ transactivation activity by increasing NF- κ B association with PPAR γ and reducing its DNA binding [105]. When the activity of PPAR γ is suppressed, mesenchymal stem cells become osteoblasts instead [106]. Therefore, NIK may indirectly contribute to osteoblast genesis.

7. The importance of NIK in cancer

7.1. Death versus survival: cIAPs and NIK

Although traditionally thought of as pro-survival factors, cIAPs have anti-proliferative and anti-survival function in B-lymphocytes due to their role in degrading NIK [31]. It is clearly demonstrated that NIK provides survival signals, especially in B-cells. For example, B-cells with TRAF2 and TRAF3 deficiency [46,74,75], as well as B-cells expressing a NIK mutant that lacks the TRAF3 domain [35], have prolonged survival due to the accumulation of NIK. B-cells from these mice become independent of CD40 or BAFFR signaling.

On the contrary, SMAC mimetic compounds, which inhibit cIAPs and stabilize NIK, are used to induce cell death in many cancer cells. The detailed mechanism of how SMAC mimetic compounds mediate cell death has been previously described [47]. These compounds potentiate auto-ubiquitination and degradation of cIAPs. As a result, both the canonical and the non-canonical pathways are activated, increasing the production of TNF α along with other chemokines such as IL-8 and MCP-1. The activation of two NF- κ B pathways cannot be inhibited by the use of a TNF-blocking agent such as TNFR1-Fc. Nevertheless, cell death induced by SMAC mimetic drugs can be inhibited by blocking TNFR signaling, implying that this signaling is necessary for cell death [47]. In support of these data, another study also observed the activation of NF- κ B (both canonical and non-canonical) and cell death in the presence of cIAPs antagonists [107]. The investigators of this study speculated that the ability of cIAP antagonists to induce the canonical NF- κ B pathway is likely due to the prolonged association of RIP-1 with TNFR when cIAPs are absent [107]. When cIAPs are degraded, NF- κ B activation may no longer be cytoprotective to counteract TNF α -induced cell death.

Another independent study regarding SMAC mimetics examined their effect on tumor growth *in vivo*. Similarly, this study also demonstrated that TNFR signaling can lead to apoptosis, induced by SMAC mimetics [108] and this cell death is also RIP-1 dependent, in agreement with the study by Vince *et al.* [107]. SMAC mimetic-induced TNF α production triggers the signaling of TNFR to form a RIP-dependent death-inducing complex in addition to its signaling through pro-survival NF- κ B activation [108]. The death signal possibly prevails over the survival function of NF- κ B, leading to cell death in cancer cells [108].

In these studies using SMAC mimetics, NIK is stabilized upon treatment with the pharmacological cIAP inhibitors, yet cell death is still induced. On one hand, NIK is thought to be a pro-survival mediator as it is a potent activator of NF- κ B. On the other hand, the role

of some of NIK negative regulators, such as TRAF2, cIAP1 and cIAP2 during the anti-apoptotic mediation of NF- κ B has been documented [34]. Although cIAPs are traditionally viewed as pro-survival factors, they antagonize the pro-survival function of NIK. Interestingly, B-cells expressing a NIK mutant with the deletion of the TRAF3 binding domain are much more susceptible to FasL-induced cell death as they have elevated Fas expression [35]. Therefore, it is possible that stabilization of either NIK or cIAPs is necessary for the survival of different cell types. NIK undergoes continuous proteosomal degradation that is executed in part by cIAPs in non-transformed cells, thus NIK may not mediate survival in this context. However, in the event of transformation, malignant cells may become dependent on the stabilization of NIK. Therefore, for the safe use of SMAC mimetics in cancers and to target NIK as a cancer therapeutic, the question of how the coordination between NIK and cIAPs favors death versus survival needs further exploration.

7.2. NIK in hematological malignancies

Mis-regulation of NIK in multiple myeloma has been well documented by several studies. Genetic screening of multiple myeloma tumor samples and cell lines suggest that many genetic mutations occur in these tumors, leading to activation of the canonical and non-canonical NF- κ B pathways. More importantly, genetic or epigenetic alterations that lead to NIK stabilization have been demonstrated in multiple myeloma [109,110]. Some negative regulators of NIK, such as TRAF2, TRAF3, cIAP1 and cIAP2, have inactivating mutations in multiple myeloma [109,110]. In addition, activating mutations have also been identified in positive regulators of NIK, such as CD40 and LT β R [109,110].

Another type of mutation is an activating mutation in NIK itself. Three of five multiple myeloma patient samples have chromosomal translocations between the NIK locus and either the IGH or IGL locus [109]. Some multiple myeloma cell lines also have chromosomal translocations or gene amplification, leading to enhanced NIK expression [109]. Furthermore, allelic deletions of TRAF3 and mutations resulting in a truncated form of TRAF3 that cannot bind to NIK were also identified [109,110]. As a result, multiple myeloma tumors from patients as well as cell lines exhibit a high NF- κ B index [109,110]. Although plasma cells, from which multiple myeloma is derived, normally display high NF- κ B activation, this activation is ligand-dependent. Therefore, it is thought that the mutations mentioned above render multiple myeloma cells with the ability to undergo ligand-independent proliferation and survival [109,110].

In support of this notion, manipulations of NF- κ B regulators alter the pro-survival phenotype. For instance, TRAF3 was re-introduced in cell lines with either bi-allelic deletions or inactivating mutations. Re-expression of TRAF3 ablates the constitutive processing of p100 to p52 as well as nuclear translocation of RelA, p50, p52 and RelB, resulting in the growth arrest of these tumor cells [109,110]. It was reported that the TRAF3 level was highly upregulated in many leukemia and lymphoma cell lines, suggesting the possibility of NIK involvement in these malignancies [111]. NIK silencing provides direct evidence for the contribution of NIK to multiple myeloma. Knocking down NIK in multiple myeloma cell lines with high NIK expression induced cell death, whereas cells with low NIK expression were unaffected [109]. These data suggest that multiple myeloma cells with elevated NIK expression are indeed dependent on NIK expression for their survival [109].

In support of the pro-survival role of NIK, expressing NIK mutant without the TRAF3 binding domain in B-cells lead to hyperplasia and independence of BAFFR signaling [35]. Since signaling from the BAFFR is crucial for B-cell survival, BAFFR-independence as a result of NIK stabilization is one of the events important for B-cell malignancy.

Despite the increasing evidence that malignancy of myeloma can be attributed to NIK, the mechanism by which NIK is mediating tumorigenesis is still elusive. The literature to date suggest that both the canonical and non-canonical pathways may be crucial for the ability of NIK to elicit transformation. shRNA-mediated depletion of NIK in multiple myeloma cell lines with TRAF3 or cIAPs mutation abrogates both canonical and non-canonical NF- κ B pathway activation [48,109]. Interestingly, silencing of IKK α using shRNA was not toxic to NIK over-expressing multiple myeloma cells, suggesting that NIK does not mediate tumorigenesis via the non-canonical pathway [109]. When NIK was over-expressed in multiple myeloma cell lines with low NF- κ B activity, the gene signature induced by NIK was almost identical to the cell lines with IKK β over-expression, implying that NIK over-expression mediated NF- κ B gene transcription to induce tumorigenesis can be through the canonical pathway [48]. However, in another cell line, the NIK over-expression-induced gene signature does not correlate well with IKK β over-expression-induced signature [48]. Hence, it remains elusive how NIK over-expression may lead to malignant cell phenotypes.

Another hematological malignancy in which NIK plays a role is T-cell leukemia and Hodgkin Reed-Sternberg cells. A genetically engineered mouse model suggests a role for NIK and NF- κ B in development of T lymphocyte malignancy. In thymocytes, NIK plays a role in Notch3 mediated NF- κ B activation in a ligand-independent manner, resulting in high proliferative and tumorigenic potential of these cells [112,113]. However, in the absence of pre-TRC/pT α , NIK does not participate in the IKK complex and p100 to p52 processing is only dependent upon IKK α [113].

In concert with the data from the mouse model, the transcript level of NIK is highly upregulated in leukemia cell lines as well as in patients [114]. Protein expression of NIK in these cells was observed only with proteasomal inhibitor, MG132, treatment suggesting that the turn-over rate of NIK is rapid in this type of tumor [114]. Although NIK knock-down did not affect cell growth *in vitro*, depletion of NIK significantly decreased tumor size in a mouse model [114]. Perhaps, NIK expression allows the tumor cells to grow in an *in vivo* tumor environment by affecting other cells in the tumor micro-environment that promote tumor growth, and this difference may not be apparent in culture where growth is more conducive or where stromal cells are absent. Although this study highlights the importance of NIK in T-cell leukemia, it remains unclear what aspect of tumor development NIK affects.

In parallel, forced expression of NIK in rat fibroblasts transformed them, as assessed by anchorage independent growth. However, the investigators of this study noted that retroviral overexpression of NIK was toxic to the cells. Thus, they used fibroblasts expressing a gene under the transcriptional control of NF- κ B, which confers resistance to an antibiotic blasticidin only when NF- κ B is constitutively active. Only under this selection pressure did NIK over-expression lead to transformation. The ability of NIK to transform these fibroblasts depends on NF- κ B activation since over-expressing the super-suppressor I κ B hindered colony formation of NIK over-expressed cells in a soft agar assay [114].

7.3. NIK in solid tumors

In addition to hematological tumors, some reports suggest the role of NIK in solid tumors, albeit less extensively. For instance, constitutive activation of the non-canonical pathway of NF- κ B as well as NIK accumulation upon MG132 treatment was demonstrated in pancreatic cancer cells [115]. When NIK expression was reduced by siRNA, cell growth and p100/p52 processing were also diminished, suggesting that those processes are attributable to NIK over-expression in these pancreatic cancer cells [115]. However, NIK does not regulate the canonical pathway in these cells [115]. Similar to pancreatic cancer cells, non-small cell lung cancers have elevated NIK expression. Depletion of NIK diminishes constitutive NF-

κ B activation of both pathways, as well as some survival or metastatic genes such as survivin and MMP-9 [116]. Therefore, depending on cancer types, NIK may regulate one or both NF- κ B pathways. Moreover, over-expression of NIK is responsible for the constitutive activation of NF- κ B in basal-like subtype breast cancer cell lines [117].

Additionally, one study suggests that NIK may be required for invasion and migration of a murine melanoma cell line [68]. In this model, OPN leads to enhanced production of MMP-9, possibly mediated by NIK activation [68]. In support of the murine melanoma model, in human melanoma cells with constitutive NF- κ B activity, there is increased association of NIK with the IKK complex in melanoma as compared to normal melanocytes based upon co-immunoprecipitation assays [118]. Interestingly, melanoma cells show higher expression of LT β R, an upstream activator of NIK. Silencing the expression of the LT β R reduces the growth and invasion of melanoma cells [119].

Although some advances have been made in understanding NIK's tumorigenic potential, additional studies are necessary to understand the possible contribution of NIK in solid tumors. Additional *in vivo* models are gravely needed to elucidate the function of NIK in carcinogenesis. Moreover, the mechanism by which NIK mediates tumorigenesis is poorly understood. NIK is likely to be affecting both NF- κ B pathways during malignancy possibly as a result of shifting the balance between the positive and negative feedback mechanisms. It cannot be excluded that NIK may have NF- κ B independent functions in contributing to tumorigenesis. As NIK is not an essential kinase for the canonical NF- κ B pathway under physiological conditions, a thorough understanding of its function may provide avenues for therapeutic interventions in cancer treatment.

8. Concluding remarks

NIK functions as a kinase crucial for the non-canonical pathway of NF- κ B and contributes to various cellular processes, ranging from the regulation of adaptive and innate immunity to cancer growth and survival. Although significant advances have been made during the past 13 years since NIK was first cloned, many questions remain unanswered. Most importantly, regulation of NIK function is not completely clear. Is stabilization of NIK required for the phosphorylation and activation of NIK? Which ligase(s) is/are responsible for ubiquitination of NIK? Although the data are suggestive that endogenous NIK is ubiquitinated under basal conditions, this has not been formally demonstrated. Answering these questions will be valuable to understand the importance of NIK in disease models such as cancer and autoimmunity, to eventually develop therapeutic interventions. NIK is an attractive therapeutic target since its function is not essential for activation of the canonical pathway of NF- κ B, though NIK can affect this pathway upon stabilization. It appears that cells under physiological conditions do not require abundant NIK expression as evidenced by the low level of NIK in most cell types and the survival of systemic NIK knock-out mice. However, in pathological contexts, such as cancer, NIK becomes stabilized. Thus, attenuating the function of NIK is a potentially promising target for cancer therapy.

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Biographies

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Figure 1.

Figure 1A. At a basal level, NIK undergoes constant proteosomal degradation, executed by four E3 ubiquitin ligases, cIAP1/2 and TRAF2/3. Under such conditions, minimal signaling emanates from NIK. **B.** Upon ligand stimulation (for example, LTβR or CD40), there are two possible processes for activation of the non-canonical pathway: 1) E3 ubiquitin ligases such as TRAF2 and 3 are degraded, leading to stabilization of NIK; 2) when the receptor is activated, it competes for TRAF2/3, thus disassembling the degradative complex from NIK. As a result, NIK becomes stabilized to perform downstream functions, including stimulation of p100 proteolysis. When NIK is stabilized by inducers of the non-canonical NF-κB pathway, amplification of the canonical NF-κB pathway can occur, even though NIK is not required for this pathway.

Figure 2.

Different domains of NIK interact with specific substrates and regulators, and are crucial for NIK function. The C-terminus of NIK mainly interacts with substrates such as IKK and p100, and NIK regulators, such as TRAF1, 2, 5 and 6. The N-terminus of NIK regulates the stability of the protein by interacting with TRAF3. So far, only one phosphorylation site (Thr-559) on NIK has been identified in its kinase domain. *Aly* mutation in the C-terminus impedes the function of NIK. NIK also has sequences for nuclear and nucleolar localization signals, which enable the protein to shuttle between different cellular compartments.

Figure 3.

The function of NIK in inducing the proteolysis of p100 to p52 is required for osteoclastogenesis. The I B function of p100 deters NF-κB family members such as RelA and RelB to translocate to the nucleus. Re-expression of RelB rescues the defective osteoclastogenesis in osteoclast precursors but RelA does not, suggesting that only RelB is required for the osteoclastogenesis.