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Atypical IκB proteins – nuclear modulators of NF-κB signaling

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Abstract

Nuclear factor κB (NF-κB) controls a multitude of physiological processes such as cell differentiation, cytokine expression, survival and proliferation. Since NF-κB governs embryogenesis, tissue homeostasis and the functions of innate and adaptive immune cells it represents one of the most important and versatile signaling networks known. Its activity is regulated via the inhibitors of NF-κB signaling, the IκB proteins. Classical IκBs, like the prototypical protein IκBα, sequester NF-κB transcription factors in the cytoplasm by masking of their nuclear localization signals (NLS). Thus, binding of NF-κB to the DNA is inhibited. The accessibility of the NLS is controlled via the degradation of IκBα. Phosphorylation of the conserved serine residues 32 and 36 leads to polyubiquitination and subsequent proteasomal degradation. This process marks the central event of canonical NF-κB activation. Once their NLS is accessible, NF-κB transcription factors translocate into the nucleus, bind to the DNA and regulate the transcription of their respective target genes. Several studies described a distinct group of atypical IκB proteins, referred to as the BCL-3 subfamily. Those atypical IκBs show entirely different sub-cellular localizations, activation kinetics and an unexpected functional diversity. First of all, their interaction with NF-κB transcription factors takes place in the nucleus in contrast to classical IκBs, whose binding to NF-κB predominantly occurs in the cytoplasm. Secondly, atypical IκBs are strongly induced after NF-κB activation, for example by LPS and IL-1β stimulation or triggering of B cell and T cell antigen receptors, but are not degraded in the first place like their conventional relatives. Finally, the interaction of atypical IκBs with DNA-associated NF-κB transcription factors can further enhance or diminish their transcriptional activity. Thus, they do not exclusively act as inhibitors of NF-κB activity. The capacity to modulate NF-κB transcription either positively or negatively, represents their most important and unique mechanistic difference to classical IκBs. Several reports revealed the importance of atypical IκB proteins for immune homeostasis and the severe consequences following their loss of function. This review summarizes insights into the physiological processes regulated by this protein class and the relevance of atypical IκB functioning.

Keywords: NF-kappaB, Atypical IkappaB proteins, BCL-3, IkappaBNS, IkappaBzeta, IkappaBL, Nuclear NF-kappaB modulation, IkappaB eta, MAIL, NFkBID

Review

NF-κB signaling

NF-κB transcription factors are homo- or hetero dimers composed of two REL proteins, such as p50 and p65 [1]. This family consists of p65/RelA, p50, p52, RelB and c-Rel. Their common structural motif is the Rel homology domain (RHD) [2]. It contains a dimerisation sequence for the interaction with other REL proteins, a nuclear localization signal (NLS) regulating its subcellular localization and a DNA binding motif for the interaction with κB sites in regulatory sequences of their respective target genes. Transactivation domains, TAD, are found in p65/RelA, c-Rel and RelB, whereby NF-κB dimers containing at least one of these subunits can induce transcription [2]. On the other hand, NF-κB homodimers of p50 and p52 function as transcriptional repressors due to the lack of such a sequence [2]. They either compete for activating NF-κB transcription factors by occupation of DNA binding sites, or recruit gene-silencing proteins such as histone deacetylases (HDACs) [3], or inhibit transcription by use of both mechanisms. Each REL-protein subunit, with its individual and slightly different DNA-binding domain, contributes to the total DNA-affinity of the

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dimeric transcription factor [4-6]. Thus, the optimal sequence for NF-κB binding is not identical among the different dimer combinations. This results in a magnitude of optimal regulatory sequences. The diversity of ideal binding sites, the multitude of κB-sites in the DNA and the existence of suppressive and inducive NF-κB dimers are the reasons of the complexity and versatility of the downstream signaling network.

NF-κB can be activated in two different fashions, called canonical and non-canonical NF-κB activation. Both pathways use a complex formed by IκB kinase proteins, however, in slightly different compositions [2,7]. Regulation of the upstream signaling events and detailed differences between the canonical and non-canonical NF-κB activation were previously illustrated and are not part of this review [2,7].

Canonical NF-κB activation

The canonical signaling is initiated by a variety of receptors, like members of the TNF receptor super-family, Toll-like receptors, interleukin receptors and antigen receptors of B and T cells [2]. Their common downstream signaling complex is a trimeric IκB kinase complex consisting of the catalytic subunits IKKα, IKKβ and the regulatory subunit IKKγ/NEMO [8,9]. The sequestration of NF-κB in the cytoplasm is mediated by the association of classical IκBs such as the prototypical protein IκBα to inhibit NF-κB binding to the DNA [10-13]. The characteristic structural motif of IκB proteins is a repetitive sequence of 6 to 10 ankyrin domains [2]. Binding of these ankyrin repeats to the REL homology domain of NF-κB results in masking of the NLS [14,15]. Crystallography demonstrated that the ankyrin domain of IκBα localizes between the carboxy-terminal Ig-like sequences of the REL homology domains of two NF-κB subunits [16]. When the NLS is accessible the NF-κB transcription factor can localize in the nucleus and bind to the DNA, which depends on IκBα degradation [17,18]. In case of IκBα this process is initiated by phosphorylation of the serine residues 32 and 36 by activated IKKβ [18-20]. The phosphorylated serines within the so-called “destruction box” of IκBα are subsequently recognized by the E3 ligase βTRCP leading to polyubiquitination and eventually causing proteasomal degradation of IκBα [17,21-23]. As the NLS of the NF-κB dimer is accessible the transcription factor localizes into the nucleus and modulates transcription via binding to the DNA.

Non-canonical NF-κB activation

The non-canonical NF-κB activation depends on an IKKα homodimer, activated for example by triggering of the BAFF receptor, CD40 or the lymphotoxin-β receptor [7,24,25]. The NF-κB dimers activated in the non-canonical signaling cascade are composed of p52 and RelB [26]. Their NLS sequences are masked intramolecularly by the precursor protein of p52, p100, which displays carboxy-terminal ankyrin repeats to interact with the REL domains and hide the NLS [27]. Phosphorylation of p100 causes cleavage of the protein into p52 leading to the nuclear translocation of NF-κB [26,28]. As the ankyrin repeats are part of the sequences of p100 as well as p105, the precursor of p50, the existence of a common evolutionary ancestor for both, IκB and REL proteins is reasonable. Alternatively, p100 and p105 could be the result of a gene fusion.

Nuclear modulation of NF-κB activity

NF-κB activity is fine-regulated in the nucleus by a variety of mechanisms, including post translational modifications of REL proteins for example sumoylation, phosphorylation, acetylation and ubiquitination [3,29]. Besides, the nuclear IκB proteins of the BCL-3 class BCL-3, $I\kappa B_{NS}$, IκBζ and IκBη can dramatically alter NF-κB-mediated effects via the regulation of dimer exchange, the recruitment of histone modifying enzymes or the stabilization of NF-κB dimers on the DNA. Although, these proteins formally belong to the IκBs due to the presence of ankyrin repeats in their structure (Figure 1), they do not functionally act exclusively as repressors of NF-κB-mediated transcription, but more as NF-κB modulators (Table 1).

BCL-3

Initial description and structure

BCL-3 was the first identified atypical IκB protein. It consists of an amino-terminal TAD followed by 7 Ankyrin repeats and a second carboxy terminal TAD, displaying an overall length of 448 amino acids (Figure 1). It was first described as a proto-oncogene expressed in patients, which suffered from B-cell chronic lymphocytic leukemia displaying the translocation t (14:19)(q32;q13.1) [30].

Function

The oncogenic potential of BCL-3 is illustrated by its capacity to dampen the tumor suppressor p53 and to force Cyclin D1 expression in order to enhance proliferation [31,32]. As the protein is expressed by a variety of different non-Hodgkin and Hodgkin lymphomas it could represent a suitable pharmacological target for the treatment of cancer [33,34]. Electrophoretic mobility shift assays initially revealed that the protein interacts with p50 and p52 and can inhibit DNA binding [32,35]. In contrast to IκBα, which sequesters p50 in the cytoplasm, BCL-3 is localized in the nucleus and alters the subnuclear localization of p50. In COS cells p50 was relatively equally distributed when overexpressed alone, however cotransfection with BCL-3 resulted in its accumulation in nuclear spots [36]. From these analyses it was thought that BCL-3 might act as an anti-repressor

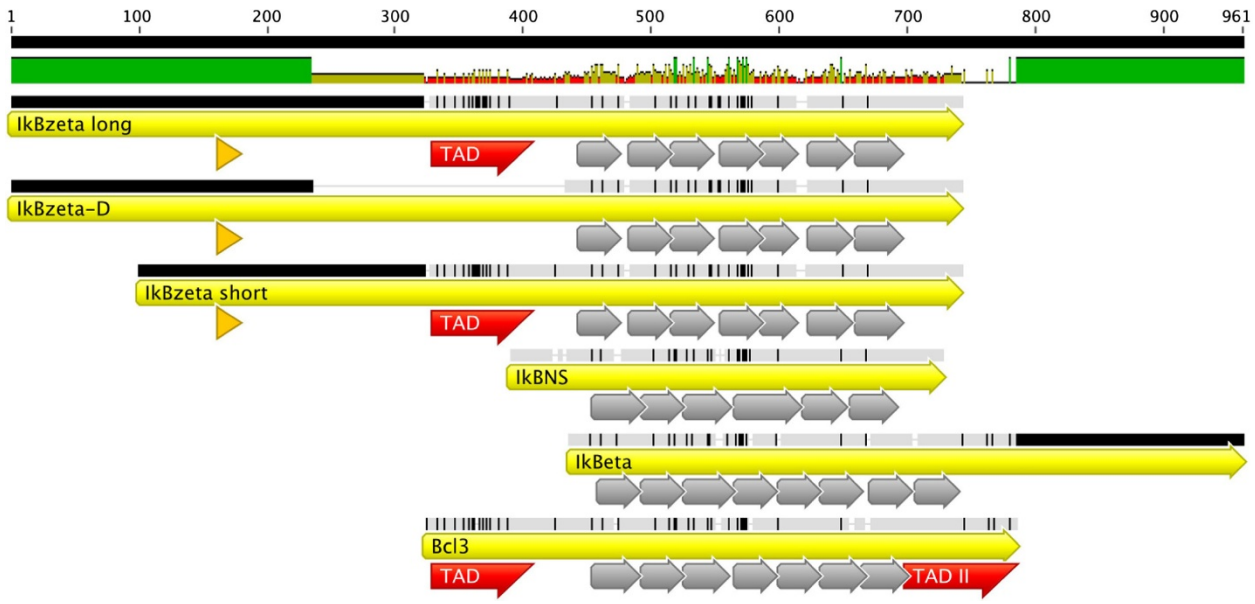

Figure 1 Alignment of atypical IκB proteins. Alignment of murine atypical IκB proteins IκBζ long, IκBζD, IκBζ short, $IκB_{NS}$, IκBη and BCL-3 is shown. Position of ankyrin repeats (grey), transactivation domains, TAD, and nuclear localization signal (orange) are indicated within the coding sequence (yellow). Identity between the sequences is indicated in the upper part of the diagram with green showing highest similarity and red lowest similarity. Sequences NP_001152867, NP_082941, NP_085115, NP_291079 and NP_742154 were used for the alignment created by Geneious v5.3 software (Drummond et al., 2010; available at www.geneious.com/).

by removing suppressive p50/p50 homodimers from the promoters of its target genes, which allows binding of activating p50/p65 or comparable heterodimers and indirectly forces transcriptional activation. Transcriptional repression of BCL-3 is also directly regulated via its binding to HDAC-1, -3 and -6 [37]. In macrophages, LPS is a potent inducer of BCL-3 [38,39], which interacts with p50 to reduce NF-κB-mediated TNF-α production. In agreement with the formation of nuclear suppressor complexes, it was suggested that this effect is mediated via chromatin remodeling. This is based on the fact that HDAC-1 overexpression further enhanced BCL-3-mediated suppression and trichostatin A treatment abrogated the BCL-3-mediated effects [39]. Alternatively, BCL-3 can suppress transcription via block of the ubiquitination of p50 to stabilize a suppressive NF-κB complex within the nucleus [38]. Thus, BCL-3-deficient macrophages display enhanced expression of pro-inflammatory cytokines upon LPS treatment, as degradation of inhibitory p50 homodimers is not blocked. Surprisingly, an early study reported that BCL-3 had the potential to induce transcription directly via an amino terminal proline rich region and a second carboxy-terminal serine/proline rich region [40]. It was later shown that its carboxy-terminal tail enhances transcription via interaction with c-Jun, c-Fos, CREB-binding protein/p300 and the steroid receptor coactivator-1 (SRC-1), with CBP/p300 and SRC-1 having acetyltransferase activity [41]. Remarkably, both the amino and carboxy terminal sequences are needed for the full transcriptional activity of BCL-3, suggesting, that both sequences function cooperatively [40]. An additional part of the nuclear BCL-3 complex is the BCL-3 binding protein B3BP thought to be involved in DNA repair, which forms a complex with p300 and BCL-3 [42]. In addition to p300, the acetyltransferase Tip60 is another interaction partner of BCL-3/p50 complexes, which can enhance transcriptional activity [43]. A recent report nicely demonstrated that p52/BCL-3 complexes bind to A/T and G/C centric NF-κB binding sites sequences, however, with a dramatically altered transcriptional outcome [44]. Via G/C centric elements these complexes induce transcription through Tip60 recruitment, but suppress via binding to A/T centric elements and recruitment of HDAC3.

BCL-3 itself is critically regulated via post-translational modifications, especially via phosphorylation and ubiquitination. It was shown that phosphorylation of BCL-3 via GSK3 regulated BCL-3 degradation and oncogenicity [37,45]. However, its proteasomal degradation in the cytoplasm is regulated by an E3-ligase complex containing TBLR1, which appears to be independently of GSK3 [46]. In all known pathways, NF-κB activity is regulated by several upstream ubiquitination events through the balance between ubiquitin ligases and deubiquitinases [2,47]. CYLD, a K63-deubiquitinase inhibits NF-κB activation in TRAF2-mediated NF-κB signaling pathways [48]. Remarkably, BCL-3 also becomes deubiquitinated by CYLD in the nucleus, upon UV-irradiation. This

Table 1 Properties of atypical IκB proteins

	BCL-3	IκBζ	$IκB_{NS}$	IκBη	IκBL
Alternative names	B-cell CLL/lymphoma 3 , D19S37, AI528691	Nfkbiz, FLJ30225, FLJ34463, INAP, MAIL, AA408868	Nfkbid, IkB-delta, MGC11314, MGC149503, TA-NFKBH, T-cell activation NFKB-like protein	Ankrd42, FLJ37874, SARP, 4931426M20, 4933417L02Rik	LST1, NF-kappa -B inhibitor-like protein 1
Chromosomal Localisation (NCBI geneID)	Human:	Human:	Human:	Human:	Human:
	19q13.1-q13.2	3p12-q12	19q13.12	11q14.1	6p21.3
	(602)	(64332)	(84807)	(338699)	(4795)
	Mouse:	Mouse:	Mouse:	Mouse:	Mouse:
	7A3; 7 9.95 cM	16; 16 C1.2-C1.3	7 B1; 7	7; 7E2	17 B1; 17
	(12051)	(80859)	(243910)	(73845)	18.6 cM (18038)
Interaction partners	p50 [35,36,40], p52 [32], c-Jun [41], c-Fos [41], CREB/p300 [41,43], SRC-1 [41], B3BP [42], Tip60 [43], HDAC-1/-3/-6 [37,45]	p50 [64], STAT3 [71], FUSS-DDIT3 [72], p65 [73], RORγ [77], RORα [77]	p50 [79], all Rel proteins (GST-pulldown) [78], cRel [80]	p50 [87]	Unknown
Tissue specific protein expression	Bone marrow, spleen, lymph nodes, peritoneal lavage, kidney, liver [52]	Heart, skeletal muscle, spleen, kidney, liver, placenta, lung, peripheral blood, leukocytes [73]	Spleen [78]	Brain, lung, kidney, testis, ovary [87]	Human PMBCs [88]
Knockout phenotype	Reduction of Peyer's Patches [53]. Lack of splenic marginal centers [51].	Dermatitis-like skin irritations [74]. Ocular surface inflammation [76]. Resistant to EAE [77].	Less Treg cells [80].	Unknown	Unknown
Induced via	LPS [39], IL-9 [54], IL-4 [57]	LPS [61], IL-1 [64], BLP [64], PGN [64], MALP [64], Flagellin [64], peptidoglycan [67], β-glucan [67], CpG-DNA [67], IL-18 [70], IL-12 [70]	LPS [79,82], CD3 [80], anti-IgM [83], CD40 [83]	LPS [87], poly(I:C) [87], CpG-DNA [87], zymosan [87]	LPS [88]
Direct target genes (ChIP, pulldown, EMSA)	IP-10 [44], IL-10 [44], MCP-1 [44], CD95 [44], CD40 [44], IL-23p19 [44], CyclinD1 [32], TNFα [39], Gata3 [55]	IL-6 [64], IL8 [72], IL-17 [77], IFNγ [70]	IL-2 [81], IL-6 [82], Foxp3 [80]	Unknown	Unknown

causes the rapid export of BCL-3 from the nucleus and its inactivation [48].

Transgenic mouse models

BCL-3 function was examined using a variety of different transgenic mouse models. Eμ-BCL-3 transgenic mice display splenomegaly, lymphadenopathy and elevated levels of mature B cells in the secondary lymphoid organs, the peritoneal cavity and the bone marrow, suggesting that BCL-3 overexpression renders B cells into a state of hyperactivation [49]. In agreement with this observation, BCL-3-deficient mice display a variety of defects in their humoral immune response. They lack germinal centers in the spleen and show impaired clearance of *Listeria, Streptococci* and *Toxoplasma* infections since they cannot mount a pathogen-specific antibody response [50-52]. Upon *Listeria* infection, reduced IL-12p70 and IFNγ levels were detected, which is presumably the result of increased levels of anti-inflammatory IL-10 produced by macrophages [50]. In addition, like p50-deficient mice, BCL-3-deficient mice display reduced Peyer´s Patches but not a complete absence of them as seen in p52/p100-deficient mice [53]. Besides the role of BCL-3 in B cells, the protein has several properties important for T cells survival and differentiation. In T cells and mast cells, BCL-3 is upregulated by IL-9 and IL-4 via the Jak/STAT pathway [54]. When BCL-3 is absent, induction of GATA-3 by IL-4 is dramatically impaired and, thus, TH2 development [55]. In contrast to this, the generation of IFNγ-producing TH1 cells is not altered in BCL-3 compromised mice [55,56]. However, the protein enhances IFNγ expression in CD8 cells upon second antigen exposure [56]. In addition, IL-4 protects cells from apoptosis via BCL-3. One report demonstrated that BCL-3 expression is lost upon IL-4 deprivation, leading to apoptosis [57]. In agreement, ectopic overexpression of BCL-3 effectively protected cells from IL-4 deprivation-induced death [57]. Consequently, it was suggested that BCL-3 could have anti-apoptotic potential. Indeed, another report demonstrated that BCL-3-deficient T cells are highly sensitive towards activation-induced cell death due to over-activated pro-apoptotic Bim [58]. In line, transgenic overexpression of BCL-3 prolonged T cell survival. In the context of T cells it was further shown, that BCL-3 in cooperation with p52 is important in regulating central tolerance [59]. However, this effect is not intrinsically mediated by T cells, but controlled by medullary thymic epithelial cells, which are required for selection of T cells. These cells display impaired maturation in BCL-3/p100 double-deficient mice, leading to severe autoimmunity [59]. In terms of autoimmune diseases it should be noted, that BCL-3 is also a suppressor of autoimmune diabetes, as BCL-3-deficient NOD mice are more susceptible to autoimmune diabetes and display higher levels of IL-17 [60].

Conclusive remarks

The protooncogene BCL-3 displays remarkable versatility in the regulation of NF-κB, for example via NF-κB stabilization in the nucleus or removal of the transription factor from the DNA. Via the recruitment of HAT and HDAC proteins BCL-3 can mediate opposing effects on transcription.

IκBζ

Initial description and structure

IκBζ was first identified by a differential display analysis in a variety of tissues upon i.p. injection of LPS in wildtype mice [61]. It was initially termed "molecule possessing ankyrin repeats induced by LPS" (MAIL), which is still a frequently used name for its murine isoforms [61]. A second study found IκBζ upon IL-1β treatment of OP9 stroma cells leading to its alternative name "interleukin-1 inducible nuclear ankyrin-repeat protein" (INAP) [62]. Up to now, its most common name is IκBζ for both the human and murine proteins [63]. IκBζ consists of a NLS (amino acids 163–178), a transactivation domain (amino acids 329–429), and seven ankyrin repeats (amino acids 450–700) (Figure 1). Early studies demonstrated the nuclear localization of IκBζ in 3T3 and OP9 cells, strong sequence homology of its Ankyrin-repeat containing C-terminal tail to BCL-3 and interaction with p50/p50 homodimers [61,62,64,65]. So far, three murine isoforms of the protein were described. Initially reported were $MAIL_L$ (728 amino acids) and the N-terminal truncated isoform $MAIL_S$ (629 amino acids) (Figure 1) [61]. Of these two isoforms, the long protein seems to be more prominently expressed [66]. The third isoform, IκBζ-D, is a splicing variant lacking amino acids 236–429 [65]. This deletion results in loss of the suggested transactivation domain (Figure 1). Consequently, IκBζ-D fails to augment NF-κB activity in contrast to the full length protein [65].

Function

IκBζ/p50/p50 complexes bind to the IL-6 locus and potentiate transcription in macrophages upon TLR-2, -4 and -9 and IL-1R triggering [64,67]. In agreement, overexpression of the downstream signaling mediators MyD88 and TRAF6 can induce IκBζ mRNA [67]. IκBζ-deficiency causes a reduction of IL-6 and of IL-12p40 expression [64], whereas its overexpression enhances IL-6 production [61]. In contrast to those two cytokines, TNFα transcription is suppressed by IκBζ, which nicely illustrates its dual functionality [65]. So far, several stimuli are known, which force the expression of IκBζ. In addition to the early identified triggers of IκBζ induction, LPS and IL-1β [67], stimulation of macrophages with peptidoglycan, β-glucan and CpG-DNA can also induce IκBζ expression [67]. On the other hand, its mRNA is not detectable upon TNFα or PMA treatment of OP9 cells

[62]. Remarkably, the promoter activity of the *Nfkbiz* gene (encoding for IκBζ) upon TNFα treatment is not markedly different compared to stimulation with IL-1β or LPS [68]. IκBζ mRNA is not detectable upon TNFα treatment alone, because it requires stabilization via IL-1β, LPS or IL-17 [68]. TNFα and IL-17 treatment in combination, however, is sufficient to induce IκBζ. Analyses of the murine IκBζ locus also revealed the presence of κB binding sites in its promoter, which suggests its regulation by NF-κB [69]. In agreement with this report, overexpression of dominant negative IκBα can prevent the induction of IκBζ by LPS treatment [67]. Interestingly, ectopic overexpression of the upstream kinases NIK and IKKβ was also sufficient to cause IκBζ-induction in contrast to overexpression of the downstream protein p65 [67].

A recent investigation addressed the function of IκBζ in NK cells. It was shown that IκBζ is induced and recruited to proximal promoter regions of the *ifng* gene upon IL-12 or IL-18 stimulation [70]. As a result of impaired NK cell activation IκBζ-deficient mice were more susceptible to MCMV infections. The effect could be pinpointed to impaired binding of STAT4 to the *ifng* locus. Remarkably, in IκBζ-deficient NK cells STAT4 phosphorylation remained unaffected [70]. Next to the regulation of STAT4, IκBζ was also reported to interact directly with STAT3 via its coiled-coiled domain [71]. Binding of IκBζ results in a dramatic reduction of the transcriptional activity of STAT3. Thereby, transcription of an anti-apoptotic target gene of STAT3, MCL-1, is impaired leading to enhanced apoptosis [71]. Another study revealed its co-localization and interaction with the nuclear fusion oncoprotein FUSS-DDIT3, originating from t(12;16)(q13;p11), which forces the development of myxoid liposarcomas [72]. It was shown that this complex binds to the *IL8* locus and thereby enhances its expression [72]. The modulation of chromatin remodeling through IκBζ was also suggested by the observation that the human protein co-localizes with HDAC-4 and HDAC-5 in nuclear spots [73]. In contrast to the murine protein, human IκBζ presumably interacts with p65 and suppresses its transcriptional activity through HDAC recruitment [73]. However, interaction studies and reporter assays were performed using ectopically overexpressed proteins in HEK 293 cells. As a consequence it still remains uncertain whether murine and human IκBζ show differences regarding the interaction with Rel proteins. It was further shown that the human protein is inducible by IL-1β and TNFα in MCF-7 and Hela cancer cells [73]. In contrast, stimulation of murine macrophages with TNFα alone was not sufficient to induce IκBζ mRNA [62,64,68], indicating that either tumor development alters IκBζ regulation or that the murine and human proteins are regulated in a different fashion. In addition, RNAi-mediated knockdown of IκBζ rendered Hela cells more resistant towards TNFα and CD95-mediated apoptosis [73]. Although certain data indicate differences in the regulation and interaction of murine and human IκBζ, further investigations need to be done to verify functional differences between the proteins of the two species.

Transgenic mouse models

IκBζ-deficient mice develop several signs of autoimmune syndromes. These comprise severe skin irritations in the face, neck and periocular regions appearing between weeks 4 and 8 after birth [74]. Further analyses revealed constitutive expression of IκBζ in keratinocytes [75]. Remarkably, its expression was not altered upon LPS treatment *in vivo* or *in vitro*, in contrast to IL-1β treatment, which enhanced IκBζ transcription. This indicates the specific repression of LPS-induced IκBζ expression in keratinocytes. Thus, IκBζ appears to be a mediator of skin homeostasis, whereby its deficiency causes a dermatitis-like phenotype. Remarkably, IκBζ is expressed in a variety of mucosal tissues, such as the ocular surface epithelium [76]. Its deficiency causes chronic inflammation of the ocular surface, leading to infiltration of $B220^+$ and $CD4^+$ cells in the submucosa. This proposes a role as negative regulator of pathologic progression of ocular surface inflammations [76]. The importance of IκBζ for adaptive immune cells was impressively demonstrated for TH17 cells. IκBζ binds, together with RORγ or RORα, to the *IL-17a* locus [77]. Their combined overexpression could enhance TH17 development from naïve T cells, even without TGFβ and IL-6 treatment. Moreover, IκBζ deficiency impairs TH17 development and results in complete resistance to experimental induced autoimmune encephalomyelitis (EAE) [77]. Thus, IκBζ could be a pharmaceutical target for the treatment of multiple sclerosis (MS).

Conclusive remarks

In summary, IκBζ can be considered a pro-inflammatory IκB protein, as it is necessary for the generation of TH17 cells and the production of IL-6 upon LPS exposure. However, as constitutive protein expression in keratinocytes prevent immune cell infiltration in the skin and IκBζ-deficient mice display signs of dermatitis, loss of the protein can also cause inflammatory syndromes.

$IκB_{NS}$

Initial description and structure

$IκB_{NS}$, also known as TA-NFKBH and Nfkbid, consists of 327 amino acids and, therefore, is the smallest member of the BCL-3 subfamily [78]. $IκB_{NS}$ was initially identified by RDA analysis, investigating genes induced upon negative selection of T cells in the thymus [78]. It consists almost entirely of six ankyrin repeats and short C- and N-terminal tails, but no transactivation

domains were reported yet (Figure 1). The interaction of $I\kappa B_{NS}$ with other NF-κB family members is not entirely clear. It was shown that overexpressed $I\kappa B_{NS}$ predominantly interacts with p50 but not p65 in RAW264.7 macrophages [79]. However, pulldown experiments using GST-$I\kappa B_{NS}$ and protein extracts from stimulated N15 TCR transgenic thymocytes demonstrated binding to cytoplasmic and nuclear p50 as well as nuclear p52, p65, RelB and c-Rel [78]. Therefore, it is conceivable that $I\kappa B_{NS}$ can interact with several different NF-κB dimers in the nucleus. One study reported mild interaction of endogenous $I\kappa B_{NS}$ and c-Rel in stimulated T cells [80]. The presence of a specific interaction might depend on posttranslational modifications and on the analyzed cell type.

Transgenic mouse models and function

The generation of $I\kappa B_{NS}$-deficient mice revealed that the protein is dispensable for negative selection, since CD4 and CD8 T cell numbers and Vβ expression are identical between $I\kappa B_{NS}$-deficient and wildtype mice [81]. Moreover, analyses of TCR specificities indicated unaltered reactivity to antigens compared to wildtype mice. However, it was shown that $I\kappa B_{NS}$ is inducible in mature CD4 T cells upon TCR stimulation [80]. Its deficiency causes reduced expression of IL-2 and IFNγ upon stimulation by anti-CD3 and anti-CD28 and mildly impaired proliferation, which could be overcome by treatment with PMA and ionomycin [81]. In $I\kappa B_{NS}$-deficient macrophages and DCs, however, LPS triggering resulted in prolonged and enhanced expression of IL-6 and IL-12p40 [79,82]. To this end it is thought that a complex containing p50 and $I\kappa B_{NS}$ is required to terminate IL-6 expression. The reductions of IL-6 and IL-12p40 on the one hand and the inductions of IL-2 and IFNγ on the other hand underline the dual function of atypical IκB proteins as repressors or inducers of transcription also for $I\kappa B_{NS}$. It is also interesting, that $I\kappa B_{NS}$ acts antagonistic to IκBζ in the regulation of IL-6 in macrophages [64,79]. Next to macrophages and T cells, a recent report suggested a role for $I\kappa B_{NS}$ in B cell development, as it is induced by LPS, anti-IgM and CD40 triggering [83]. Notably, $I\kappa B_{NS}$-deficient mice lack the entire B1 B cell compartment and display reduced B cell numbers in the marginal zone [83,84]. Corresponding to impaired T cell proliferation upon TCR triggering, proliferation was reduced upon LPS and anti-CD40 triggering in $I\kappa B_{NS}$-deficient B cells [83]. In agreement with the impaired generation of plasma cells *in vitro*, serum IgM and IgG3 levels were dramatically reduced and less antigen-specific antibodies were produced upon *influenza* infection of $I\kappa B_{NS}$-deficient mice. Remarkably, one report demonstrated, that $I\kappa B_{NS}$ expression is suppressed by an AP-1/Foxp3 complex [85]. Foxp3 governs the generation and function of immunosuppressive regulatory T cells. Of note, IL-2 secretion in Treg cells is prevented. Thus, it is conceivable that $I\kappa B_{NS}$ repression might ensure silencing of IL-2 transcription in Treg cells, as it is needed for IL-2 induction upon activation of CD4 and CD8 cells [81]. Although the protein is repressed in $Foxp3^+$ Tregs, $I\kappa B_{NS}$ is important for the maturation of $Foxp3^-$ Treg precursors [80]. Thus, Treg cells are reduced in $I\kappa B_{NS}$-deficient mice. Whether or not human $I\kappa B_{NS}$ functions in a similar fashion remains unknown. The sole report on human $I\kappa B_{NS}$ demonstrated that its mRNA is induced upon IL-1β treatment of immortalized human gingival fibroblasts, along with the other NF-κB proteins p50, p52, p65, RelB IκBα, IκBε, and IκBζ [86].

Conclusive remarks

$I\kappa B_{NS}$ is necessary for the generation of immunosuppressive Treg cells and the termination of pro-inflammatory cytokines like IL-6 and IL12p40. On the other hand it promotes germinal center reactions and IL-2 induction. Thus, the protein mediates immune activation as well as suppression. Therefore, it is an important regulator of immune homeostasis, although it cannot simply be classified as a pro- nor anti-inflammatory signaling protein.

IκBη

IκBη is the most recently identified member of the BCL-3 subfamily, found by microarray analyses of bone marrow derived DCs [87]. It was shown that the protein made up of 516 amino acids is induced upon LPS, polyI:C, CpG DNA and zymosan treatment in RAW264.7 macrophages [87]. In contrast to the other BCL-3 proteins, it consists of 8 ankyrin domains and a prolonged carboxy terminal tail (Figure 1). Co-Immunoprecipitation experiments demonstrated its interaction with p50, but not with p65. Its siRNA-mediated knockdown led to the loss of the expression of several pro-inflammatory genes, such as the classical NF-κB target genes *Il6*, *Il1b* and *ifnb* [87]. In agreement with the reduced expression of cytokines upon IκBη loss, its overexpression mediated increased luciferase activity of NF-κB consensus constructs [87]. The obvious functional similarity to IκBζ suggests redundancy of the two proteins, but the prolonged carboxy terminal tail is unique to IκBη (Figure 1). Generation of IκBη-deficient mice is essential to further determine functional differences or redundancies between the two proteins.

IκBL

It is still a matter of debate, whether the two reported IκBL isoforms, α(L) and α(S), belong to the group of IκB proteins, because no REL protein was identified as an interaction partner so far. Nevertheless, the protein contains ankyrin repeats, is localized in the nucleus and suppresses NF-κB target genes TNFα and IL-6 [88]. Furthermore, fluorescent microscopy revealed its localisation in nuclear

dot-like structures [89], a property of BCL-3 [36], IκBζ [73], IκBη [87] as well as $IκB_{NS}$ (unpublished data). Although both reports strongly indicate the identification of an additional nuclear IκB protein, interaction with an NF-κB subunit is a prerequisite to consider IκBL part of this class.

Conclusions

The BCL-3 subfamily of IκB proteins alters NF-κB activity in a positive or negative fashion. BCL-3, IκBζ, $IκB_{NS}$ and IκBη exhibit their function in the nucleus, via association with NF-κB subunits on the DNA. Their main interaction partners are p50 and p52 within the NF-κB pathway [32,36,62,78,87]. The observed interaction of overexpressed human IκBζ with p65 and interaction studies using GST-$IκB_{NS}$ and *in vitro* translated REL proteins suggest, that atypical IκB proteins can also bind to the other NF-κB subunits [73,78]. However, these interactions might be cell type specific and could depend on specific stimuli or posttranslational modifications of IκBs and REL proteins as well. Atypical IκBs exert their transcriptional function by a magnitude of mechanisms, whose regulations and interplay are not completely understood. BCL-3 stabilizes p50 homodimers on the DNA to silence specific genes because their capacity to compete with activating p50/p65 heterodimers is increased. On the other hand, it is also possible that BCL-3 removes p50 homodimers and relocalizes them to the nucleus in dot-like structures that are associated with HDAC proteins in order to repress transcription [37]. In both examples, BCL-3 acts as a factor, which regulates the maintenance of NF-κB binding to the DNA. Remarkably, atypical IκBs can also recruit proteins, which alter transcription via changes of the chromatin structure. BCL-3 interaction with the histone acetyl transferases p300 and Tip60 [42,43], as well as co-localization of IκBζ with HDAC4 and HDAC5 was observed using confocal microscopy [73]. Apparently the interaction with chromatin remodeling enzymes is a dynamic process, as BCL-3 does not exclusively bind to acetyl transferases, but can also co-localize with HDAC proteins in the nucleus. Further analyses of $IκB_{NS}$ and IκBη are needed to determine, whether recruitment of histone modifying enzymes is a mechanism common to all atypical IκB proteins.

Remarkably, all atypical IκBs are induced via LPS stimulation (Table 1) [39,61,79,87]. Although the sequence similarities of the proteins is high (Figure 2), it remains unknown, how and if atypical IκB proteins cooperate or compete with each other during the regulation of common target genes and common interaction partners like p50. As an example, IκBη and IκBζ were both shown to force IL-6 production in macrophages, as loss of these proteins shortened the expression period and the level of the secreted cytokines [64,87]. These data suggest a cooperative function. Nevertheless, it is unknown, whether this depends on direct protein interaction between the two IκBs or whether it is mediated via two different κB binding sites, or via two different dimers, which are sequentially exchanged. On the other hand, IL-6 expression is repressed by $IκB_{NS}$, since its loss prolongs the period of cytokine secretion and increases their expression level [79,82]. Thus, $IκB_{NS}$ acts in an opposite fashion to IκBζ and IκBη. It is highly likely, that these proteins are sequentially recruited to the IL-6 locus, to regulate the induction and termination of cytokine expression. However, comprehensive studies are needed, to verify this hypothesis.

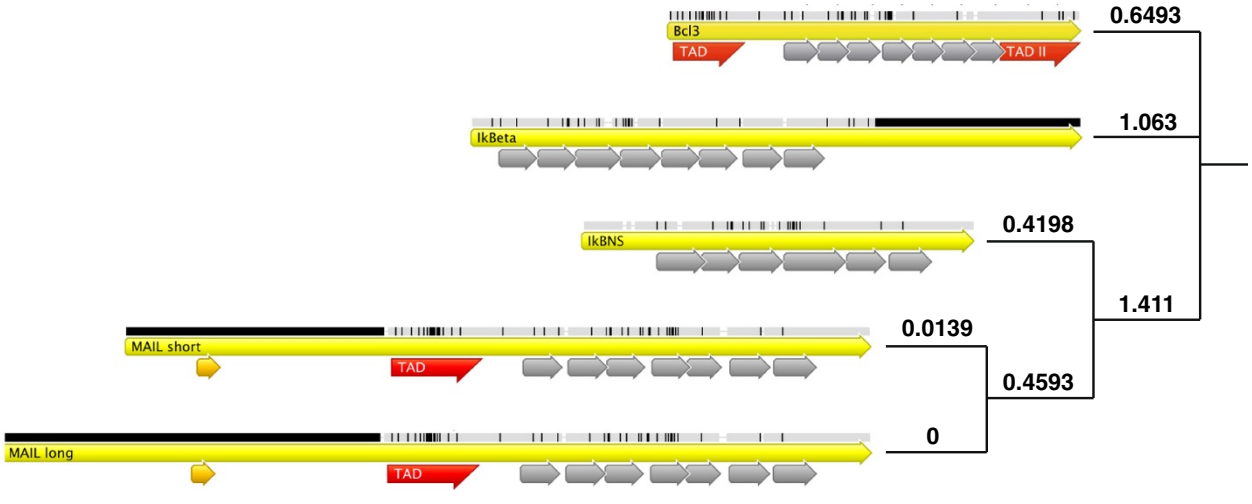

Figure 2 Homology of atypical IκB proteins. Homology tree between the atypical IκB proteins IκBζ long, IκBζ short, $IκB_{NS}$, IκBη and BCL-3 is shown. Numbers indicate evolutionary distance between the different proteins. Tree was generated by Geneious v5.3 software (Drummond et al., 2010; available at www.geneious.com/).

Several reports demonstrated the oncogenic potential of BCL-3, as the protein is highly upregulated in a variety of cancer cells, suppresses the activity of p53 and acts in an anti-apoptotic fashion [31-33]. Thus, the analyses of the BCL-3 expression status might be suitable for determining the prognosis of tumor progression and the disease course. It might also represent a suitable pharmacological target for cancer treatment. Atypical IκBs are of particular importance for the development of distinct T helper cell subsets. BCL-3 is an essential mediator of TH2 development via GATA-3 upregulation and IL-4 secretion, without affecting the TH1 subset [55]. IκBζ-deficient mice are completely protected from EAE as IκBζ-deficient T cells fail to develop into IL-17 producing TH17 cells [77]. At last, $I\kappa B_{NS}$ drives the development of regulatory T cells by binding to regulatory elements of the Foxp3 locus, whereby $I\kappa B_{NS}$-deficient mice display reduced Treg numbers [80]. Thus, there exists compelling evidence that atypical IκBs are specific regulators of T helper cell subsets. Therefore, pharmacological targeting of atypical IκBs might help to develop therapies to treat diseases, which depend on a certain T cell subset. As atypical IκBs are involved in a variety of cellular processes, but the understanding of their molecular regulation and relationship remains incomplete, further studies are necessary to uncover their pharmacological potential in the future.

Abbreviations

AP-1: Activator protein 1; BCL-3: B-cell lymphoma 3-encoded protein; CD: Cluster of differentiation; ChIP: Chromatin Immunoprecipitation; CREB: cAMP response element-binding protein; CtBP: C-terminal-binding protein 1; CYLD: Cylindromatosis; DNA: Deoxyribonucleic acid; EAE: Experimantal induced autoimmune encephalomyelitis; Foxp3: Forkhead box protein 3; GSK3: Glycogen synthase kinase 3; GST: Glutathione S-transferase; HAT: Histone acetyl transferase; HDAC: Histone deacetylase; IFNγ: Interferon gamma; Ig: Immunoglobuline; IκB: Inhibtior of NF-κB; IKK: IκB Kinase; IL: Interleukin; INAP: Interleukin-1 inducible nuclear ankyrin-repeat protein; JAK: Janus kinase; LPS: Lipopolysaccharide; LSD1: Lysine-specific demethylase 1; MAIL: Molecule possessing ankyrin repeats induced by LPS; MCMV: Murine cytomegaly virus; MS: Multiple Sclerosis; MyD88: Myeloid differentiation primary response gene (88); NCOR: Nuclear receptor co-repressor; NEMO: NF-κB essential modulator; NF-κB: Nuclear factor kappa B; NIK: NF-κB inducing kinase; NK: Natural killer; NLS: Nuclear localization signal; NOD: Non-obese diabetic; RDA: Representational difference analysis; RHD: REL homology domain; RNA: Ribonucleic acid; RAR: Retinoic acid receptor; ROR: RAR-related orphan receptor; SRC-1: Steroid receptor coactivator 1; STAT: Signal transducer and activator of transcription; TAD: Transactivation domain; TNFα: Tumor necrosis factor alpha; TRAF2: TNF receptor associated factor.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

All authors contributed in the conception and writing of the manuscript. All authors edited and approved the final version.

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