Atypical I κ B proteins in immune cell differentiation and function

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Keywords: NF-kappaB, atypical IkappaB, immune system, macrophage, T cell

Abbreviation: ARD, Ankyrin repeat domain; ANK, ankyrin repeats; Bcl-3, B cell lymphoma 3 protein; EAE, experimental autoimmune encephalomyelitis; ERK, Extracellular-signal-regulated kinase; FO, follicular; GC, germinal centre; I κ B, inhibitor of NF- κ B; NF- κ B, nuclear factor kappa-light-chain-enhancer of activated B cells; LPS, lipopolysaccharide; MZ, marginal zone, NLS, nuclear localization sequence; PP, Peyer's Patches; RHD, Rel homology domain; TAD, transcription activation domain; TCR, T cell receptor; TLR, toll like receptor

Abstract

The NF- κ B/Rel signalling pathway plays a crucial role in numerous biological processes, including innate and adaptive immunity. NF- κ B is a family of transcription factors, whose activity is regulated by the inhibitors of NF- κ B (I κ B). The I κ B proteins comprise two distinct groups, the classical (cytoplasmic) and the atypical (nuclear) I κ B proteins. Although the cytoplasmic regulation of NF- κ B is well characterised, its nuclear regulation mechanisms remain marginally elucidated. However, work from recent years indicated that nuclear I κ Bs contribute significantly to the modulation of NF- κ B-mediated transcription in the immune system. Here, we discuss the role of the atypical I κ B proteins Bcl-3, I κ B ζ , I κ B_{NS}, I κ B η and I κ BL for the regulation of gene expression and effector functions in immune cells.

1.1 Introduction to the NF- κ B and I κ B protein families

The basis for today's knowledge about nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) was its discovery in 1986 as a regulator of κ B light chain expression in B cells [1]. Since that time, the function of the NF- κ B transcription factor family was investigated in many research areas and the knowledge of NF- κ B expanded rapidly involving the regulation of gene expression, cellular processes and diseases. The NF- κ B family (also named Rel family) comprises 5 members termed p50/105, p52/100, p65 (RelA), c-Rel and RelB. They are characterised by an N-terminal Rel homology domain (RHD), which regulates dimerization and DNA-binding [2–4]. NF- κ B family members form homo- or heterodimers. Depending on the presence of a transcription activation domain (TAD) those dimers are able to activate or inhibit the expression of target genes [2–4]. Thus, due to their TAD homo- or heterodimers containing p65 (RelA), c-Rel or RelB enable the recruitment of co-activators and the expression of target genes whereas homo- or heterodimers of either p50 or p52 are thought to repress transcription (they lack a TAD). This is fine-tuned further by intracellular regulators of NF- κ B. In non-activated cells, NF- κ B dimers are located in the cytoplasm, where they become functionally inactivated by classical members of the inhibitor of NF- κ B (I κ B) family, I κ B α , I κ B β and I κ B ϵ (Figure 1) [5]. These I κ B proteins bind to the RHD of NF- κ B and mask the nuclear localization sequence (NLS) within the RHD, preventing the nuclear localization of NF- κ B dimers [5]. Upon cell activation, for example via the T cell receptor (TCR), the I κ B kinase complex becomes activated and phosphorylates the NF- κ B-bound I κ B protein inducing proteasomal degradation of the phosphorylated I κ B protein [5,6]. Thereupon, functionally active NF- κ B dimers translocate into the nucleus and bind to specific κ B-sites at the DNA. Each of the typical, cytoplasmic I κ B proteins exhibits a diverse affinity to a specific NF- κ B dimer and different kinetics of degradation, thus, enlarging the regulatory capacity of the five NF- κ B members [3,5].

An ankyrin repeat domain (ARD), containing six to eight single ankyrin repeats (ANK), is the common structural motif of all I κ Bs (Table 1 and Figure 1) [5]. Each ANK comprises 33 amino acids folded into a helix-loop-helix conformation [5]. The ARD is essential for protein stability and interaction with the RHDs of NF- κ B dimers. N-terminal to the ARD, classical I κ Bs exhibit an unfolded structure with a signal response domain (also called degron motif), containing serine residues for stimulation-dependent phosphorylation by IKK [3,5,6]. Furthermore, N-terminal to the phosphorylation sites of typical I κ B proteins, lysine residues are located where polyubiquitination occurs. Both typical I κ B proteins, I κ B α and I κ B β have a region called PEST (rich in proline, glutamic acid, serine and threonine), which has been suggested to mediate fast protein turnover [5]. The NF- κ B family members p50 and p52 originate from the precursors p105 and p100, respectively, which become post-translationally cleaved. Both, p105 and p100 contain C-terminal NF- κ B-inhibiting ANKs as well as an N-terminal Rel homology domain. Consequently, they act both as NF- κ B and as I κ B protein [5,7].

Classically, I κ B proteins retain NF- κ B dimers within the cytoplasm and thus, were defined as inhibitors of NF- κ B. However, in 1992, the discovery of the atypical I κ B protein Bcl-3 diversified this group of proteins [8–11]. Besides this protein, I κ B ζ , I κ B_{NS}, I κ B η and I κ BL belong to the atypical or nuclear I κ B proteins (Figure 1) [12–16]. Both, cytoplasmic and nuclear I κ B proteins are characterized by ARDs, as mentioned before. Next to their nuclear localization, atypical I κ B proteins are further distinguished from typical I κ Bs by exhibiting a distinct kinetic of protein expression. Upon stimulation with NF- κ B-inducing agents, cytoplasmic I κ Bs are degraded whereas the levels of nuclear I κ Bs are increased [5]. Interestingly, nuclear I κ Bs can inhibit target gene expression or act as enhancers for specific gene expression. Consequently, atypical I κ B proteins represent a later NF- κ B-regulatory

element that comes into place after cell activation to take over the regulation of NF- κ B dimers from the cytoplasmic I κ B proteins. Thus, atypical I κ B proteins have the potential to fine-tune NF- κ B activation and its transcriptional responses. Due to the heterogeneous activity of the atypical I κ B proteins this review will highlight their function within major classes of immune cells (summarized in Table 2 and Figure 2).

1.2 The members of the atypical, nuclear I κ B protein family

Bcl-3, I κ B ζ , I κ B_{NS}, I κ B η and I κ BL belong to the atypical, nuclear I κ B proteins (Table 1 and Figure 1). Mice deficient for a single nuclear I κ B protein grow normally after birth, but, several defects of their organ structure and/or immune system were observed. Thus, mice deficient for nuclear I κ B proteins suffer from autoimmune diseases and/ or show altered defence towards several pathogens, which will be outlined below.

1.2.1 Bcl-3

Bcl-3 (B-cell lymphoma 3 protein) was first identified as a proto-oncogene in patients suffering from B cell chronic lymphocytic leukaemia [17] and was later shown to interact with the NF- κ B subunits p50 and p52 (Table 1) [12,18,19]. The expression of the seven ANK-containing protein is induced upon stimulation with lipopolysaccharide (LPS), IL-1 β , IL-4, IL-9, IL-10, IL-12 [20–26]. Bcl-3-deficient mice show a defect of splenic architecture, characterized by the absence of germinal centres within the white pulp of the spleen [27,28]. Beside the absence of germinal centres, intestinal Peyer's Patches (PP) are reduced in numbers [29]. Furthermore, Bcl-3-deficient mice are defective in clearing *Listeria monocytogenes* [27] and die of *Streptococci pneumonia* (associated with acute sepsis) [27] and *Toxoplasma gondii* infections [28,30]. In addition, Bcl-3-deficient mice do not suffer

from spontaneous autoimmunity, but are more susceptible to the induction of type 1 diabetes [31].

1.2.2 I κ B ζ

I κ B ζ (also known as INAP, MAIL or NFKBIZ) contains six ANK and its expression was first detected in several murine organs upon LPS-injection [13]. The expression of I κ B ζ is up-regulated by B cell receptor crosslinking as well as stimulation with β -glucan, Toll-like receptor- (TLR) ligands (CpG-DNA, bacterial lipoprotein, peptidoglycan, flagellin, MALP-2, R-848), TGF β -1, IL-1 β , IL-4, IL-12, IL-18 and IL-17 plus TNF- α [32–38]. Like Bcl-3, I κ B ζ interacts with the NF- κ B member p50 and additionally with p65 [33,34,39]. Mice defective for I κ B ζ grow normally after birth but almost 90% of I κ B ζ -deficient animals die during embryogenesis and adult mice suffer from spontaneous autoimmunity [34,40]. I κ B ζ -deficient mice develop an atopic dermatitis-like disease 10 weeks after birth as well as conjunctivitis [34,40,41]. Furthermore, I κ B ζ -deficient mice are resistant to EAE (experimental autoimmune encephalomyelitis) induction upon immunisation with myelin oligodendrocyte glycoprotein (MOG)-peptide [42] but are highly susceptible to MCMV infections [38].

1.2.3 I κ B_{NS}

The seven ANK-containing I κ B_{NS} (alternative names are NFKBID or TA-NFKBH) was first identified in studies of antigen-induced negative selection [14]. Similarly to peptides triggering negative selection, I κ B_{NS} expression is induced by LPS, IL-10, anti-IgM, CD40 and TCR activation [14,43–47]. GST-tagged I κ B_{NS} was shown to interact with all NF- κ B subunits in a pulldown analysis using thymic lysates [14]. Further analysis verified the interaction with p50 in RAW macrophages [44], as well as p50 and c-Rel in regulatory T (Treg) cells [43]. I κ B_{NS}-deficient animals breed and grow normally. Two independent I κ B_{NS}-

deficient mouse lines were investigated for germinal centre (GC) formation with differing results. More specifically, sheep red blood cell immunisation resulted in defective GC formation in $\text{I}\kappa\text{B}_{\text{NS}}$ -deficient animals [47] whereas normal GC formation was observed upon immunisation with 4-Hydroxy-3-nitrophenylacetyl-Chicken Gamma Globulin (NP-CGG) [48]. In addition, the knockout of $\text{I}\kappa\text{B}_{\text{NS}}$ increased the susceptibility of mice to infection with *Citrobacter rodentium* [49] and influenza A virus [47]. Although $\text{I}\kappa\text{B}_{\text{NS}}$ -deficient mice do not suffer from spontaneous autoimmunity and are more resistant to EAE induction [50], transfer of $\text{I}\kappa\text{B}_{\text{NS}}$ -deficient T cells to RAG1-deficient animals resulted in an increased colitis [43,49].

1.2.4 $\text{I}\kappa\text{B}\eta$ and $\text{I}\kappa\text{BL}$

The function of the recently identified members of the nuclear $\text{I}\kappa\text{B}$ family, $\text{I}\kappa\text{B}\eta$ and $\text{I}\kappa\text{BL}$, is largely unknown. $\text{I}\kappa\text{B}\eta$ (alternative names are ANKRD42 or SARP) is an eight ANK-containing protein identified in gene expression analysis of bone marrow-derived DC [15,51]. Knockdown studies revealed a function of $\text{I}\kappa\text{B}\eta$ in proinflammatory cytokine expression. In contrast to other $\text{I}\kappa\text{B}$ proteins, $\text{I}\kappa\text{B}\eta$ is constitutively expressed, while LPS stimulation triggers a slight induction. Like other $\text{I}\kappa\text{Bs}$, $\text{I}\kappa\text{B}\eta$ interacts with the NF- κB member p50 [15]. Whether the LPS-inducible $\text{I}\kappa\text{BL}$ (also known as NFKBIL1 or LIST) belongs to the $\text{I}\kappa\text{B}$ protein family is still under debate. Since $\text{I}\kappa\text{BL}$ contains two ANKs [16,52] and is localised within the nucleus [53] it was suggested that it belongs to nuclear $\text{I}\kappa\text{Bs}$ but an interaction with NF- κB dimers was excluded [54]. Nevertheless, polymorphisms within the gene locus of $\text{I}\kappa\text{BL}$ are implicated in systemic lupus erythematosus, primary Sjögren's syndrome, ulcerative colitis and rheumatoid arthritis [54–56].

1.3 The atypical I κ B protein family in macrophages

Macrophages are a crucial cell type of the innate immune system. These phagocytes engulf and digest pathogenic microorganisms, cell debris, foreign substances and degenerated cells and link innate and adaptive immunity. The following section deals with the function of nuclear I κ B proteins in macrophages (Table 1 and Figure 2A).

1.3.1 Bcl-3 in macrophages

Gram-negative bacteria express LPS as a major component of their cell wall. LPS is one of the strongest activators of macrophages and monocytes. LPS-stimulation of macrophages induces expression of pro-inflammatory cytokines including IL-6, IL-12 and TNF- α . Upon LPS stimulation, Bcl-3-defective peritoneal macrophages exhibit an increased expression of TNF- α . Accordingly lentiviral overexpression of Bcl-3 in RAW macrophages reduced the expression of TNF- α [22,24,57]. Mechanistically, LPS caused the expression of IL-10 and this cytokine induced the expression of Bcl-3. Thereupon, Bcl-3/p50 binds to the TNF- α promoter and competes with the binding of transcription activating RelA and c-Rel [24]. Accordingly, RelA and c-Rel are bound to the TNF- α promoter in LPS-activated Bcl-3-deficient macrophages [57]. Furthermore, Bcl-3 is not only expressed in a IL-10-dependent manner but also acts on IL-10 gene expression [22,57,58]. However, whether Bcl-3 inhibits or induces the expression of IL-10 is still controversially debated. Experiments with bone marrow-derived macrophages by Wessells and colleagues indicated an IL-10-expression supporting function and thereby an anti-inflammatory effect of Bcl-3 [22]. Nonetheless, publications by Carmody et al., Riemann et al. and Pène et al. showed the opposite result [57–59]. Carmody and colleagues analysed the effect of LPS-stimulation in bone marrow derived macrophages, but revealed a negative regulation of IL-10 by Bcl-3 [57]. Whether these drastic differences arise from the strength of the LPS signal (different LPS concentration used) has to

be further analysed. In the report by Riemann et al. Bcl-3 was shown to regulate IL-10 by inhibiting its gene expression in LPS-stimulated peritoneal macrophages [58]. Furthermore, studies on the activation of alveolar macrophages with LPS or heat-inactivated *Klebsiella pneumoniae* supported the assumption of an inhibitory effect of Bcl-3 to IL-10, but it has to be considered that LPS stimulation did not affect the expression of TNF- α in the Bcl-3-deficient macrophages [59]. The expression of Bcl-3 was suggested to depend on the transcription factor STAT3. Hence, STAT3-deficient peritoneal macrophages did not induce Bcl-3 expression with or without IL-10 stimulation [24]. LPS stimulation of Bcl-3-deficient macrophages increased the level of the cytokine IL-1 β and some chemokines (CXCL1, CXCL2, CCL2, CCL5 and CCL9), compared to wildtype cells [57]. Macrophages and their LPS-induced cytokines are essential in septic shock. In line with the ability of Bcl-3 to block LPS-induced cytokine expression by macrophages, wildtype mice reconstituted with Bcl-3-deficient bone marrow are more sensitive to septic shock [57]. The splenic architecture of Bcl-3-deficient animals is disrupted and the macrophage population in the marginal zone of the spleen is at least partially lost, similarly to p52-deficient mice [28,60]. As mentioned above, Bcl-3 is able to bind to the p52 subunit of NF- κ B. In human cytomegalovirus infection, macrophages are the cells predominantly infiltrating the infected organ, but are also one of the most infected cell types. Macrophages up-regulate Bcl-3 expression and interaction with p52 upon human cytomegalovirus infection [61]. Subsequently, the Bcl-3/p52 complex binds to the major immediate-early promoter (MIEP) of human cytomegalovirus and was suggested to regulate MIEP expression [61].

1.3.2 I κ B ζ in macrophages

Like Bcl-3, I κ B ζ is induced by LPS-stimulation of macrophages [33]. Three I κ B ζ splicing variants, namely I κ B ζ (S) also called INAP, I κ B ζ (L) and I κ B ζ (D) have been described

[13,62–64]. Both, I κ B ζ (S) and I κ B ζ (L) own a TAD whereas I κ B ζ (D) lacks this domain [64]. Peritoneal macrophages predominantly express the splicing variant I κ B ζ (L) [65]. On the one hand, I κ B ζ is another nuclear I κ B protein that inhibits LPS-induced expression of TNF- α in macrophages [64]. On the other hand, p50-bound I κ B ζ enhances the expression of IL-6 via association to its promoter, in contrast to Bcl-3, which does not regulate IL-6 [34,64,66,67]. The binding sites of NF- κ B and cEBP β in the *IL6* promoter were suggested to be essential for I κ B ζ -dependent IL-6 protein expression [66]. Additionally, I κ B ζ regulates IL-12p40 expression by binding to its promoter [67]. Bcl-3 and I κ B ζ have an antagonistic effect to CCL2 expression in macrophages. While Bcl-3 inhibits the expression of CCL2, I κ B ζ promotes the expression of this chemokine by interaction with its promoter and induction of transcription [57,68]. Furthermore, the LPS-induced mRNA expression of GM-CSF and G-CSF in peritoneal macrophages strongly depends on I κ B ζ [34]. Extracellular-signal-regulated kinase (ERK) inhibition in macrophages, did not affect the expression of I κ B ζ , indicating that the ERK-pathway is dispensable for I κ B ζ expression [69]. Furthermore, it was suggested that I κ B ζ is an essential regulator for the tri-methylation of H3K4 (Histone 3 Lysine 4), which is an epigenetic marker of transcriptionally active chromatin [67].

1.3.3 I κ B_{NS} in macrophages

I κ B_{NS} was shown to be expressed by macrophages of the colonic lamina propria and the peritoneum. I κ B_{NS} is an essential negative regulator of the MyD88-dependent cytokines IL-6, IL-12p40 and IL-18 upon LPS-stimulation [44,45]. In contrast to I κ B ζ , I κ B_{NS} regulates LPS-induced expression of IL-6 and IL-12p40 in macrophages in a negative manner while it is dispensable for TNF- α expression in macrophages [44,45]. These effects are mediated by the interaction of a p50/I κ B_{NS} complex with the IL-6 but not the TNF- α promoter [44]. The

analysis of basal protein expression of peritoneal and lamina propria macrophages indicated $\text{I}\kappa\text{B}_{\text{NS}}$ - as well as Bcl-3-dependent suppression of inflammatory responses in the intestine. Whereas peritoneal macrophages do not express these two nuclear $\text{I}\kappa\text{B}$ proteins before LPS-stimulation, lamina propria macrophages exhibit basal expression, which might suppress excessive intestinal inflammatory cytokines expression [44]. In a model of septic shock, $\text{I}\kappa\text{B}_{\text{NS}}$ -deficient animals showed significantly increased expression of IL-6 and IL-12p40, resulting in exacerbated lethality, similarly to Bcl-3-deficient mice [45]. Further investigations demonstrated the dispensability of $\text{I}\kappa\text{B}_{\text{NS}}$ for the movement of macrophages toward injured arteries [70].

1.3.4 $\text{I}\kappa\text{B}\eta$ and $\text{I}\kappa\text{BL}$ in macrophages

As mentioned above, splenic macrophages exhibited basal expression of $\text{I}\kappa\text{B}\eta$, whereas LPS-stimulation increased the $\text{I}\kappa\text{B}\eta$ -expression only slightly, compared to the previously described nuclear $\text{I}\kappa\text{Bs}$. In addition, $\text{I}\kappa\text{B}\eta$ regulated the expression of pro-inflammatory cytokines, similarly to Bcl-3, $\text{I}\kappa\text{B}\zeta$ and $\text{I}\kappa\text{B}_{\text{NS}}$. Moreover, the expression of LPS-induced IL-1 β , IL-6, TNF- α , G-CSF and GM-CSF was reduced upon the knockdown of $\text{I}\kappa\text{B}\eta$ in macrophage cell lines [15].

Like $\text{I}\kappa\text{B}\eta$, $\text{I}\kappa\text{BL}$ is expressed by RAW macrophages at basal levels and LPS stimulation slightly increased this expression. Furthermore, $\text{I}\kappa\text{BL}$ exhibited an inhibitory effect towards LPS-induced expression of IL-6 and TNF- α but did not regulate the expression of IL-1 β in macrophages [52].

1.4 Atypical I κ B proteins in other innate immune cells

Besides macrophages, more innate immune cells respond to infection in an early immune phase. In this section we sum up the knowledge of nuclear I κ Bs in dendritic cells (DC), mast cells, neutrophils and natural killer cells (NK).

1.4.1 Bcl-3 in innate immune cells

Bcl-3 affects phagocytes, DCs and neutrophils. Similar to macrophages, LPS was shown to induce the expression of Bcl-3 in DCs [71]. Bcl-3-deficient mice do not have GC and their follicular DCs do not form the typical clusters observed in wildtype GC. Furthermore, follicular DCs are reduced in number whereas the numbers of myeloid DCs are increased but disorderly distributed [28,29]. In bone marrow-derived DCs LPS-induced Bcl-3 suppresses the expression of IL-23p19, an essential cytokine in T helper 17 (T_H17) cell expansion and survival [31,72]. Further analysis of bone marrow-derived DCs revealed that Bcl-3 inhibits the LPS-induced mRNA expression of IL-1 β , IL-6 and TNF- α as observed in macrophages [57]. In contrast, an analysis of cytokine secretion by Bcl-3-deficient bone marrow-derived DCs showed unaltered secretion of IL-6, IL-10, IL-12p70 and TNF- α [73]. Moreover, conditional CD11c⁺-specific (Bcl-3-ADC) or conventional Bcl-3 knockout mice were infected with *T. gondii* and cytokine secretion was measured. IL-12p40 and IL-12p70 expression was unaltered three days after infection but, was reduced after a week [30]. In addition, Bcl-3-ADC as well as Bcl-3-deficient mice were highly susceptible to *T. gondii* infection. In this regard, Bcl-3 was suggested to be dispensable in the early innate response by DCs but essential in DC-dependent activation and priming of the adaptive T cell response [30]. Furthermore, survival, maturation and expansion of DCs was supported by Bcl-3 [73]. Upon *Klebsiella pneumoniae* infection of Bcl-3-deficient mice an increased number of neutrophils was recruited to the infected lungs, presumably due to an increased expression of the

neutrophil-attracting chemokines CXCL1 and CXCL2. Nevertheless, Bcl-3-defective mice were much more susceptible to *K. pneumoniae* infection. While the bacterial uptake by neutrophils did not depend on Bcl-3 but inhibited intracellular killing [59]. Bcl-3 expression in mast cells was induced by IL-4 as well as IL-9 stimulation and presumably depends on Jak/STAT signalling [20]. In *T. gondii* infection Bcl-3-deficient NK cells showed a normal expression of IFN- γ at least in the early immune response [30]. Besides, preservation of cytotoxic activity of Bcl-3-defective NK cells was observed in *T. gondii* infection [28].

1.4.2 I κ B ζ in innate immune cells

I κ B ζ -deficient mice exhibited reduced infiltration of monocytes in a model of peritonitis, which was suggested to arise from reduced expression of the monocyte-chemotactic CCL2 [68]. IL-1 β or TLR-signalling in murine and human monocytes induces the expression of I κ B ζ [39,66,74]. IL-6 expression by human monocytes depends on the binding of p50/I κ B ζ to its promoter. Furthermore, the expression of I κ B ζ was higher in monocytes compared to monocyte-derived macrophages, which was in turn reflected in lower IL-6 expression in macrophages compared to monocytes [66]. Furthermore, I κ B ζ -induced IL-6 expression not only depended on NF- κ B but also on cEBP β and AP-1 [66]. In addition to monocytes, I κ B ζ was expressed in DCs and LPS-induced IL-6 expression was reduced in I κ B ζ -defective conventional DCs [42]. Additionally, during late phase of LPS stimulation I κ B ζ was shown to mediate the targeting of the *Il6*-promoter by the ten-eleven translocation-2 protein and thus the repression of the IL-6 gene. [75]. In DCs, β -glucan enhanced the expression of IL-1 β . Both IL-1 β as well as β -glucan activated human DCs and were crucial in maintenance of high I κ B ζ levels [36]. I κ B ζ itself was essential for optimal late gene expression, which in turn was necessary for DC migration, adhesion, production of antimicrobials and T_H17-polarising

molecules [36]. In contrast to IL-1 β , IFN- γ blocks I κ B ζ expression in β -glucan-activated DCs and consequently the T_H17-priming cytokines IL-6 and IL23 [36].

The expression of I κ B ζ by NK cells was induced upon activation with IL-12 plus IL-18. Furthermore, I κ B ζ itself was essential for IL-12/IL-18-induced gene expression and NK cell cytotoxic activity, but not NK cell development. In that respect, IL-12 plus IL-18-induced IFN- γ expression was depended on I κ B ζ because I κ B ζ promoted the binding of STAT4 to conserved non-coding elements of *Ifng* promoter [38]. Additionally, I κ B ζ bound to the promoter of INF- γ via association to p50/p65 [74].

1.4.3 I κ B_{NS} in innate immune cells

I κ B_{NS} is dispensable for surface expression of MHC-II or the co-stimulatory ligand CD86 in LPS stimulated bone marrow-derived DCs [45]. Additionally, I κ B_{NS} was not involved in TNF- α and IL-10 production, but is crucial for IL-6, IL-12p40 and IL-12p70 expression in bone marrow-derived DCs [45]. Compared to conventional DCs, regulatory DCs produced reduced levels of pro-inflammatory cytokines but increased levels of IL-10, thereby reducing serum levels of pro-inflammatory cytokines that are produced by LPS-stimulated macrophages. Furthermore, regulatory DCs showed increased expression of I κ B_{NS} as well as Bcl-3 compared to conventional DCs, whereas I κ B_{NS} was expressed 2-fold higher than Bcl-3 [76]. To which extent both I κ B_{NS} and Bcl-3 are involved in the suppression of pro-inflammatory cytokines and the induction of IL-10 will be a subject of future studies.

Like I κ B ζ , I κ B_{NS} was shown to be essential for IFN- γ production by NK cells, but dispensable for their cytotoxic activity. Furthermore, I κ B_{NS}-deficient mice do not suffer from defective NK cell differentiation [46].

1.4.4 4 I κ B η and I κ BL in innate immune cells

Beside macrophages, I κ B η is expressed in DCs, but until now the functional role of I κ B η in DCs is not known [15]. In transgenic mice human I κ BL was expressed in DCs inhibiting the expression of co-stimulators and the production of cytokines (IL-2, IL-6 and TNF- α) upon LPS-stimulation. In line with these observations, human I κ BL expressing DCs are less efficient to induce T cell differentiation [77].

1.5 Atypical I κ B proteins in lymphocytes of the adaptive immune system

The adaptive immune response is essential for specifically combating pathogenic invaders and for generating memory for a faster immune reaction to a subsequent second infection. B- and T-lymphocytes facilitate the defence against pathogens, which overcome the first barriers of the immune system and their precise regulation is crucial to circumvent autoimmunity. The function of nuclear I κ B proteins in T cells shown in this section is summarized in Table 1 and Figure 1B.

1.5.1 Bcl-3 in adaptive immunity

Bcl-3 was shown to be essential in the development of a proper architecture of secondary lymphoid organs as well as effective adaptive immunity. The splenic architecture of Bcl-3-deficient mice is defective, with absence of germinal centres and distinct B cell follicular areas [27,28]. Furthermore, intestinal PP show structural changes and are reduced in number as well as in size [27,29]. High Bcl-3 expression was found in B cell follicular areas of the spleen [60]. Moreover, Bcl-3-deficient mice show a reduction in the frequencies and absolute numbers of follicular (FO) B cells [29,78]. The disruption of the microarchitecture of PP and spleen is reflected in defective adaptive immune responses, since Bcl-3-deficient mice are more susceptible to infections with *L. monocytogenes*, *S. pneumoniae*, *T. gondii* and influenza

[27,28]. Bcl-3 is dispensable for immunoglobulin production in the steady state and upon infection. However, generation of pathogen-specific antibodies was highly diminished in the absence of Bcl-3 [27,28]. Furthermore, Bcl-3 is essential in T cell-dependent antibody responses, since the GC reaction upon immunisation with the model antigen 2,4,6-Trinitrophenyl-keyhole limpet hemocyanin (TNP-KLH) immunisation is impaired in Bcl-3-deficient animals [28]. In contrast to the reduced number of FO B cells, Bcl-3-deficient mice exhibit an increase of marginal zone (MZ) B cells, whereas a B cell-specific overexpression of Bcl-3 induces a lack of MZ B cells in mice [78,79]. In both, FO and MZ B cells surface expression levels of MHC-II, CD40, CD54, CD69, CD86 as well as the α L-subunit of the β 2-integrin LFA-1 were increased upon loss of Bcl-3. Elevated expression of the LFA-1 subunit was suggested to increase retention of MZ B cells in Bcl-3-deficient marginal zones [78]. Although the proliferation of Bcl-3-deficient B cells is elevated upon LPS-stimulation compared to wildtype cells, the proliferation of FO B cell is reduced upon anti-IgM treatment [57,78]. An enhanced number of Bcl-3-deficient FO B cells is positive for activated caspase-3, suggesting that Bcl-3 supports FO B cell survival [78]. In contrast to B cells, the frequency of splenic T cells was almost unaltered in Bcl-3-deficient mice and PP contained more CD8⁺ T cells [29,80]. In T cells, Bcl-3 acts as a survival signal but is dispensable for the regulation of proliferation, cell cycle and IL-2 expression [71,80–82]. Bcl-3 expression was shown to be induced by IL-4, with AP-1 and AP-1-like proteins being essential for IL-4-induced Bcl-3 promoter activity [21]. As reported for mast cells, IL-9 stimulation also induces Bcl-3 expression in T cells in a Jak/STAT-dependent manner [20]. In CD8⁺ T cells, Bcl-3 is crucial for maximal IFN- γ production upon a second antigen treatment, whereas it is dispensable for IFN- γ production in CD4⁺ cell [83]. Furthermore, CD8⁺ T cells require three signals for efficient clonal expansion, full activation and memory establishment. Besides antigen recognition and co-stimulation, IL-12 is an essential signal in CD8⁺ T cells and IL-12

promoted CD8⁺ T cell survival in a Bcl-3-specific manner [25,83]. Bcl-3 was shown to support T_H2 cells, since p50/Bcl-3 complex binds and transactivates the T_H2 master transcription factor GATA3 and thus promotes T_H2 differentiation [84]. Bcl-3-deficient mice were resistant to T cell transfer model of colitis and EAE induction. This resistance was mediated by reduction of IFN- γ ⁺ T_H1 cells but an increase of IL-17⁺ cells [85]. Nevertheless, Bcl-3 is not important for T_H1 differentiation and expression of the master transcription factor T-bet [84]. Furthermore, Bcl-3-deficient T_H1 cells proliferated normally, although more T_H1 cells convert to T_H17-like cells. Thus, Bcl-3 was suggested to promote the T_H1 phenotype [85]. In contrast to Bcl-3-defective NK cells having normal cytotoxic activity, cytotoxic T lymphocyte activity was reduced in *T. gondii* infected Bcl-3-deficient mice [28]. In summary, Bcl-3 regulates differentiation, survival and effector functions of various B cell and T cell subsets.

1.5.2 I κ B ζ in adaptive immunity

I κ B ζ is expressed by Treg, T_H1, T_H2 and T_H17 cells, with T_H17 cells exhibiting the highest expression level [42]. T_H17 cells mainly express I κ B ζ (L) and low levels of I κ B ζ (S), but not I κ B ζ (D) [42]. I κ B ζ expression in CD4⁺ cells does not depend on MyD-88 or the T_H17 master transcription factor ROR γ t, but is regulated by STAT3 [42]. Additionally, CD4⁺ T cell proliferation, the *in vitro* differentiation of T_H1 and T_H2 cells and the *in vivo* development of Treg cells were shown to be independent of I κ B ζ . Conversely, T_H17 cell development is regulated by I κ B ζ [42]. At this point it has to be mentioned that the two cytokines that are essential in T_H17 cell differentiation, IL-6 and TGF- β , induce the expression of I κ B ζ [42]. Furthermore, I κ B ζ -deficient cells exhibited a reduced mRNA expression of the T_H17-related genes *Il17a*, *Il21*, *Il22* and *Il23r* [42]. I κ B ζ was recruited to the promoter or enhancer region of these genes. In this respect, in T_H17-primed cells I κ B ζ , ROR γ t as well as ROR α

cooperated in IL-17A expression. I κ B ζ binds to the promoter of IL-17A in a ROR γ t-dependent and ROR α -dependent manner and was suggested to enhance the binding of transcriptional co-activators to drive *Il17a* expression [42]. Thus, I κ B ζ -deficient mice exhibited an almost absent T_H17 response while the T_H1 response was not affected in EAE [42]. Additionally, EAE was induced after adoptive transfer of CD4⁺ cells into RAG2-deficient mice and animals reconstituted with I κ B ζ -deficient cells were resistant, revealing a T cell-intrinsic effect of I κ B ζ [42]. Furthermore, mRNA of I κ B ζ was shown to become degraded by the RNase Regnase-1 (also termed as MCPIP1), which impaired the expression of IL-17-dependent genes [86,87]. In contrast, a T cell-specific disruption of I κ B ζ revealed an inhibitory effect of I κ B ζ in T_H1 and Treg cell differentiation. Hence, I κ B ζ ^{Lck} mice exhibited an increased expression of IFN γ ⁺ and FoxP3⁺ cells in the periphery [88]. It was suggested that I κ B ζ negatively regulates the activity of the IFN- γ promoter [88]. Furthermore, a Treg-specific knockout of I κ B ζ showed that Treg cell plasticity and stability was autonomous from I κ B ζ , whereas their immune-regulatory function depended on I κ B ζ [88]. Moreover, analysis of the effect of I κ B ζ in Treg differentiation revealed the inhibition of the FoxP3 promoter by I κ B ζ [37]. Thus, speculations arose, whether I κ B ζ is essential in maintaining T_H cell homeostasis [37,88].

The expression of I κ B ζ is induced upon B cell stimulation via the B cell receptor (BCR), TLR7 or TLR9, whereat the BCR and TLRs act synergistically [35,89]. Furthermore, I κ B ζ signalling in B cells is essential for TLR-induced but not BCR-induced *Il10* and *Ctla4* gene induction as well as B cell proliferation. In contrast, TLR- but not BCR-induced secretion of TNF- α as well as the up-regulation of CD86 is inhibited by I κ B ζ [35]. In addition, B cell maturation is not affected by I κ B ζ -deficiency [35]. I κ B ζ is further associated with T cell-independent type 1 humoral responses, given that IgM and IgG3 levels were reduced upon immunisation with TNP-LPS, but not upon TNP-Ficoll (type 2) immunisation. This defect in

T cell-independent type 1 humoral responses of B cells was hypothesised to arise from defective differentiation to plasma cells and class switch recombination after TLR signalling [35].

1.5.3 I κ B_{NS} in adaptive immunity

I κ B_{NS} is expressed by T cell subsets such as T_H1, T_H17 and Treg precursor cells [43,46,49]. Its expression is upregulated in the thymus by signals triggering negative selection, as well as the TCR in the periphery [14,43]. CD4⁺ and CD8⁺ T cells and thymocytes deficient for I κ B_{NS} are defective in proliferation upon TCR stimulation, but were shown to proliferate like wildtype cells after the addition of IL-2 or IL-7 [45,46]. I κ B_{NS} regulates the expression of IL-2 in lymphocytes and LN-derived CD8⁺ T cells. The regulation of IL-2 by I κ B_{NS} is mediated via the NF- κ B binding site within the IL-2 gene locus. Furthermore, IFN γ production by I κ B_{NS}-deficient lymphocytes and LN-derived CD8⁺ T cells was reduced [46]. Nevertheless, the function of I κ B_{NS} in T_H1 differentiation is far from being clear. In experimental models of colitis, either induced by the chemical compound dextran sodium sulphate (DSS), which disrupts the epithelial barrier, or by transfer of naïve T cells into lymphopenic mice, increased expression of IFN γ was detected in splenic CD4⁺ T cells (DSS colitis) or colon of I κ B_{NS}-deficient T cell recipients (transfer colitis) [43,45]. Furthermore, after transfer of I κ B_{NS}-deficient T cell to RAG1-defective mice, recipient mice showed at least a mild increase of IFN γ ⁺ CD4⁺ T cells [43,49]. In contrast, induction of EAE in I κ B_{NS}-defective mice did not alter the T_H1 cell subset [50]. Additionally, *Citrobacter rodentium* infection of I κ B_{NS}-deficient mice induced a reduction of IFN γ ⁺ CD4⁺ T cells in the spleen but no differences were detectable in the colon [49]. However, efficient *in vitro* polarisation of IFN γ ⁺ T cells from naïve T cells and proliferation of T_H1-polarised cells depended on I κ B_{NS} [49]. Besides T_H1 cells, *in vitro* and *in vivo* T_H17 polarisation and proliferation depended on I κ B_{NS}. In

Citrobacter rodentium infection and EAE, as well as colitis induction the number of IL-17⁺ T cells was severely diminished upon the loss of IκB_{NS} [49,50]. The expression of several cytokines was dependent on IκB_{NS}, since IL-10, IL-17A, IL-17F and GM-CSF expression was impaired in IκB_{NS}-deficient T_H17 cells [49,50]. Furthermore, CD4⁺ IκB_{NS}-deficient cells were defective in T_H17-related RORγt and CCR6 expression, which, at least in part, may explain their defect in cytokine secretion. However, direct regulation of the *Il17a* gene locus by IκB_{NS} was excluded [50]. In contrast, a chromatin immunoprecipitation showed the binding of IκB_{NS} to the *Il10* gene locus, hence IL-10 is directly regulated by IκB_{NS} [49]. Since the IκB_{NS} protein itself does not contain a DNA-binding domain, it is currently unclear via which transcription factor IκB_{NS} is recruited to the *Il10* locus. Future experiments have to show to which NF-κB dimer IκB_{NS} binds at the *Il10* gene or whether other transcription factors mediate IκB_{NS} recruitment.

IκB_{NS}-deficient mice exhibit a roughly 50% reduction in regulatory T cells [43]. Surprisingly, apoptosis was not affected by IκB_{NS} [43]. Furthermore, in contrast to the reduced proliferation of IκB_{NS}-deficient T_H1 and T_H17 cells, peripheral Treg cells from IκB_{NS}-deficient mice exhibited increased proliferation in the steady-state [43]. Instead, the reduced Treg cell numbers were due to a defect in Treg development, since IκB_{NS} mediated the transition of Treg precursors into mature Treg cells by driving the expression of FoxP3 [43]. In this regard, IκB_{NS} binds together with p50 and c-Rel to the promoter and the conserved non-coding sequence-3 within the FoxP3 gene locus [43]. On the other hand, after the induction of FoxP3 IκB_{NS} gene expression is suppressed by the transcription factor FoxP3 itself [43,90]. Accordingly, IκB_{NS} is dispensable for the regulation of Treg cell functionality, since IκB_{NS}-deficient Treg cells are as efficient in suppression of conventional cells as their wildtype counterparts [43].

Besides T cells, $\text{I}\kappa\text{B}_{\text{NS}}$ is an essential regulator of B cell development and function. Mice deficient for $\text{I}\kappa\text{B}_{\text{NS}}$ are defective in the development of B1 B cells and MZ B cells and the differentiation to plasma cells. Furthermore, the humoral immune response is deregulated. Although $\text{I}\kappa\text{B}_{\text{NS}}$ -deficient B cells exhibit increased surface levels of IgM, the serum levels of some immunoglobulins (IgM, IgG3) are reduced [47,48]. Moreover, the $\text{I}\kappa\text{B}_{\text{NS}}$ -deficient animals were further impaired to mounting a T cell-dependent humoral immune response, as well as in generating antigen-specific immunoglobulins and exhibit a defective IgG3 class switch [47]. Finally, the proliferation of $\text{I}\kappa\text{B}_{\text{NS}}$ -deficient B cells upon LPS or anti-CD40 stimulation was decreased, while was not affected upon BCR-stimulation [47,48].

1.5.34 $\text{I}\kappa\text{B}\eta$ and $\text{I}\kappa\text{BL}$ in adaptive immunity

Until now little is known about the function of $\text{I}\kappa\text{B}\eta$ and $\text{I}\kappa\text{BL}$ in lymphocytes. $\text{I}\kappa\text{B}\eta$ is known to be expressed in T and B cells [15]. $\text{I}\kappa\text{BL}$ was shown to be expressed in T cells in rheumatoid synovial tissue [54]. Overexpression of $\text{I}\kappa\text{BL}$ in T cells uncovered a suppressor function of $\text{I}\kappa\text{B}\eta$ in the exon exclusion during the alternative splicing of CD45 [91]. Additionally, transgenic expression of human $\text{I}\kappa\text{BL}$ increased the proliferation and IL-2 expression of murine T cells [77]. Clearly, further studies are necessary to identify distinct functions of $\text{I}\kappa\text{B}\eta$ and $\text{I}\kappa\text{BL}$.

1.6 Conclusions

The discovery of the reciprocal effect of the transcription factor NF- κB and its regulators, the $\text{I}\kappa\text{B}$ -proteins, is an essential step forward towards understanding gene regulation during infections and inflammatory processes. Previous studies revealed that nuclear $\text{I}\kappa\text{B}$ -proteins are essential for survival, clearance of infections and for protection from autoimmunity. Nuclear $\text{I}\kappa\text{B}$ -proteins regulate differentiation, maturation and activation of immune cells and

thus, expression of their surface markers, cytokines, chemokines and other effector proteins (like immunoglobulins). In short, the whole immune reaction is guided by the regulation of NF- κ B dimers via nuclear and cytoplasmic I κ B proteins. Nevertheless, further investigations are necessary to illuminate the specific regulation of a particular disease by nuclear I κ Bs. Consequently, by gaining more insights into the biology of nuclear I κ Bs we might develop novel strategies to treat infections or autoimmune diseases much better in the future. An initial step is the work of Collins and colleagues who designed a peptide mimicking the functional activity of the ANK1 of Bcl-3 (amino acid 144- 158) [43]. To this end, they fused the Bcl-3-derived peptide with the HIV-tat cargo carrying sequence and the resulting peptide was named BDP2. The BDP2 peptide was able to inhibit TLR-induced cytokine expression *in vitro*, as well as strongly reduced carrageenan-induced oedema formation and production of the cytokines IL-6 and TNF- α [43]. Thus, nuclear I κ B proteins are promising targets for the development of new improved drugs for the therapy of autoimmunity and infections, however their pharmacological relevance needs to be revealed.

Acknowledgements

K. K.-D. was supported by the President's Initiative and Networking Fund of the Helmholtz Association of German Research Centers (HGF) under contract number VH-GS-202. This work was supported by grants from the the Deutsche Forschungsgemeinschaft SFB854, project B12 to S.K. and B.S., as well as SFB 854, project A23N, SCHM1586/6-1 and the Fritz Thyssen Stiftung to I.S..

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Figure 1: Schematic illustration of the IκB protein family. IκB proteins are subdivided according to their location into cytoplasmic and nuclear IκBs. Their common feature is the existence of an ankyrin repeat domain (ARD), composed of six to eight single ankyrin repeats (ANK).

Figure 2: Known functions of nuclear IκB proteins in macrophages and T cells. In macrophages (A) the expression of all nuclear IκB is induced by LPS-stimulation. In T cells (B) nuclear IκB protein expression was shown to be induced by divers stimuli. (A-B) Nuclear IκB proteins are shown in white circles and their particular interaction partners are illustrated in non-coloured circles. Arrows indicate inhibitory (red) or stimulatory (blue) effects. IκBη and IκBL are constitutively expressed and dotted arrows designate their partial induction. Prolif., proliferation.

Table 1: General characteristics of nuclear I κ B proteins.

	Alternative protein name	Gene name	Ankyrin repeats	NF-κB specificity
Bcl-3	B cell CLL/lymphoma 3 protein	<i>Bcl-3</i>	7 [17]	p50, p52 [12,18,19]
IκBζ	INAP, MAIL, NFKBIZ	<i>Nfkbiz</i>	6 [13]	p50, p65 [33,34,39]
IκB_{NS}	NFKBID, TA-NFKBH	<i>Nfkbid</i>	7 [14]	p50 in RAW cells [44], c-Rel and p65 in Treg [43], p65, RelB and c-Rel [14]
IκBη	ANKRD42, SARP	<i>Ankrd42</i>	8 [15]	p50 [15]
IκBL	LIST1, NFKBIL1	<i>Nfkbil1</i>	2 [16,52]	unknown

Table 2: Main targets of nuclear I κ B proteins.

	Macrophages	DCs	NK	T cells	B cell
Bcl-3	TNF α * [22,24,57]; IL-10 [22,57–59]; IL-1 β , CXCL1, CXCL2, CCL2, CCL5, CCL9 [57]; MIEP of HCMV [61]	IL-12p19 [31,72]; IL-1 β , IL-6, TNF- α [57]; IL-12p40, IL-12p70 [30];		IFN- γ [83]	
IκBζ	TNF- α [64]; IL-6* [34,64,66,67]; IL-12p40* [67]; CCL2* [68]; GM-CSF, G-CSF [34]; H3K4 [67]	IL-6* [36,75]; IL-23 [36]	IFN- γ * [38,74]	IL-17A*, IL-21*, IL-22*, IL-23R* [42]; IFN- γ [88]	IL-10, CTLA4, TNF- α , CD86, IgM, IgG3 [35]
IκBη s	IL-6*, IL-12p40, IL-18 [44,45]	IL-6, IL-12p40, IL-12p70 [45]	IFN- γ [46]	IL-2, IFN- γ [46]; IL-10*, IL-17A, IL-17F, GM-CSF, ROR γ t, CCR6 [49,50]; FoxP3* [43]	IgM, IgG3 [47,48]
IκBη	IL-1 β , IL-6, TNF- α , G-CSF, GM-CSF [15]			CD45 [91]	
IκBL	IL-6, TNF- α [52]	IL-2, IL-6, TNF- α [77]		IL-2 [77]	

Marked (*) proteins were shown to be regulated by interaction of the respective I κ B protein with their gene locus.