The Atypical Inhibitor of NF-κB, IκBζ, Controls Macrophage Interleukin-10 Expression

Sebastian Hörber†, Dominic G. Hildebrand‡, Wolfgang S. Lieb‡, Sebastian Lorscheid‡, Stephan Hailfinger‡, Klaus Schulze-Osthoff§, and Frank Essmann‡

From the †Interfaculty Institute of Biochemistry, Department of Molecular Medicine, University of Tübingen, 72076 Tübingen, Germany and the §German Cancer Consortium (DKTK) and German Cancer Research Center, 69120 Heidelberg, Germany

Macrophages constitute a first line of pathogen defense by triggering a number of inflammatory responses and the secretion of various pro-inflammatory cytokines. Recently, we and others found that IκBζ, an atypical IκB family member and transcriptional coactivator of selected NF-κB target genes, is essential for macrophage expression of a subset of pro-inflammatory cytokines, such as IL-6, IL-12, and CCL2. Despite defective pro-inflammatory cytokine expression, however, IκBζ-deficient mice develop symptoms of chronic inflammation. To elucidate this discrepancy, we analyzed a regulatory role of IκBζ for the expression of anti-inflammatory cytokines and identified IκBζ as an essential activator of IL-10 expression. LPS-challenged peritoneal and bone marrow-derived macrophages from IκBζ-deficient mice revealed strongly decreased transcription and secretion of IL-10 compared with wild-type mice. Moreover, ectopic expression of IκBζ was sufficient to stimulate IL10 transcription. On the molecular level, IκBζ directly activated the IL10 promoter at a proximal κB site and was required for the transcription-enhancing trimethylation of histone 3 at lysine 4. Together, our findings show for the first time the IκBζ-dependent expression of an anti-inflammatory cytokine that is crucial in controlling immune responses.

For macrophages, two distinct states of polarization have been defined. Classically activated (M1-polarized) macrophages exert their pro-inflammatory role as effector cells in cell-mediated immune responses, whereas alternatively activated (M2-polarized) macrophages are involved in immunosuppression, wound healing, and tissue regeneration (4). In contrast to M1 macrophages, the M2 counterparts secrete high amounts of anti-inflammatory cytokines, including the crucial immunosuppressive cytokine IL-10, thereby guaranteeing a balanced immune response (5).

IL-10 can inhibit various macrophage functions, such as nitric oxide synthesis and pro-inflammatory cytokine production, as well as the expression of major histocompatibility complex proteins and co-stimulatory receptors (6, 7). In contrast, the absence of IL-10 results in spontaneous development of inflammatory bowel disease and increased pathological alterations caused by uncontrolled responses to infectious pathogens (8, 9). IL-10 may also act as a negative feedback regulator of chronic infectious diseases by inhibiting IL-6, IL-12, and TNFα secretion, thereby keeping immune responses in check and preventing tissue damage (9). Furthermore, the administration of exogenous IL-10 has been shown to ameliorate inflammatory and autoimmune diseases in several animal models (8).

An important regulator for the expression of cytokines and other immune regulators is the transcription factor NF-κB. The NFκB family consists of five members that bind as homo- or heterodimers at κB sites in the DNA of target genes (10). Depending on their transactivation activity, the NFκB subunits can be divided into two subgroups. RelA (p65), RelB, and c-Rel possess a C-terminal transcription activation domain, whereas p50 (Nkb1) and p52 (Nkb2) lack a transcription activation domain. Based on these structural differences, NFκB dimers containing at least one subunit with a transcription activation domain act as transcriptional activators, whereas p50/p50 or p52/p52 homodimers are assumed to function as transcriptional repressors.

Because various stimuli activate the NFκB signaling pathway and a great diversity of target genes is regulated by NFκB, a precise control of NFκB activity is required to avoid misguided immune responses. In fact, NFκB activation is controlled by a series of cytosolic and nuclear regulatory events, in which IκB proteins play a pivotal role (10, 11). In unstimulated cells, NFκB is sequestered as an inactive complex bound to cytosolic IκB proteins such as IκBα, IκBβ, and IκBε. Various stimuli cause the phosphorylation of cytosolic IκBs, leading to...
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their proteasomal degradation, which subsequently enables NF-κB to translocate to the nucleus and activate target genes.

Despite the presence of high-affinity binding sites, only a fraction of NF-κB target genes is generally activated in response to an inflammatory stimulus. It was suggested that NF-κB target genes can be categorized in two groups based on their kinetics of induction and the requirement of protein synthesis (12, 13). Although primary NF-κB response genes are rapidly induced, the expression of secondary target genes is delayed and requires the prior synthesis of additional NF-κB coregulators. A novel and emerging group of such NF-κB coregulators consists of the nuclear factor of kappa light polypeptide gene enhancer in B-cells (NF-κB). The atypical NF-κB protein IkBζ has been recently implicated in the regulation of anti-inflammatory gene products.

**Experimental Procedures**

**Animals—Nfkbzf−/−** and control C57BL/6 mice were used at 6–8 weeks of age as described previously (23). Nfkbzf−/− mice were originally generated by injection of targeted AB2.2 ES cell (129 strain) clones into C57BL/6 murine blastocysts (24) and were backcrossed in a C57BL/6 background for more than 50 generations. Mouse work was performed in accordance with the German law guidelines of animal care as permitted by regional authorities (Regierungspräsidium Tübingen, application no. H6/12).

**Culture of Peritoneal and Bone Marrow Macrophages—** Female C57BL/6 mice were euthanized by CO₂ asphyxiation. For isolation of peritoneal macrophages (PMΦ), the abdominal skin was removed, a catheter (24-gauge) was inserted into the peritoneal cavity, and 10 ml of ice-cold PBS was injected. After massage of the peritoneum, peritoneal fluids were aspirated and centrifuged at 500 × g for 10 min, and the resulting PMΦ were resuspended in 2 ml of macrophage medium containing DMEM/Ham’s F-12, 10% FCS, and MycoZapPlusCL antibiotics (Lonza, Basel, Switzerland). Cells were seeded in 96-well plates and cultured for 2 h under standard conditions (5% CO₂, 37 °C). Next, adherent PMΦ were washed four times with culture medium to remove non-adherent cells. To generate bone marrow-derived macrophages (BMMΦ), the femur and tibia were separated at the knee joint and rinsed with PBS. Bone marrow cells were singularized (40-μm cell strainer) and pelleted by centrifugation (500 × g, 10 min). Cells were resuspended in macrophage medium supplemented with M-CSF (30 ng/ml, Immunotools, Friesoythe, Germany), seeded in tissue culture flasks (3 × 10⁵ cells/ml), and cultured under low-oxygen conditions (5% CO₂, 5% O₂). After 7 days of differentiation, cells were washed with PBS, scrapped off, and cultured at a density of 2 × 10⁵ cells/cm² under low-oxygen conditions.

**Activation and Pro-inflammatory Stimulation of PMΦ and BMMΦ—** PMΦ and BMMΦ were cultured with murine IL-4 (100 ng/ml) or IFNγ (25 ng/ml, both from Immunotools) for 24 h to induce alternative or classical macrophage activation. Pro-inflammatory stimulation was achieved by culturing cells for the indicated time in the presence of 1 μg/ml LPS (Escherichia coli serotype O111:B4; Sigma–Aldrich, Taufkirchen, Germany). Recombinant murine IL-10 was obtained from Immunotools.

**Culture of Raw264.7 Cells, Raw264.7/TetOn-IkBζ Cells, and MEFs—** Mouse embryonic fibroblasts (MEFs) were isolated at embryonic day 10.5 according to standard procedures. MEFs and Raw264.7 cells were cultured in DMEM/high glucose, 10% FCS, and MycoZapPlusCL (Lonza). Raw264.7/TetOn-IkBζ cells were described previously (15) and cultured in the presence of 1 mg/ml neomycin (G418, Lonza). Cells were seeded at a density of 10⁵ cells/cm² 24 h before the experiment. Option ally, 2 μg/ml doxycycline (Sigma–Aldrich) was added to promote ectopic IkBζ expression. For pro-inflammatory stimulation, cells were treated with 1 μg/ml LPS.

**Transfections and Reporter Gene Assays—** Raw264.7 or Raw264.7/TetOn-IkBζ cells were transfected with appropriate amounts of plasmids using jetPEI transfection reagent according to the instructions of the manufacturer (Polyplus, Illkirch, France). Expression plasmids for Nfkb1 (p50) and IkBζ have been described previously (15, 17). For reporter gene assays, the following luciferase promoter constructs were used: pGL2basic-II10 and truncation mutants (28), pGL3basic-Lcn2 (29), and pGL3basic-II6 and pGL3basic-Elam1 (16). Cells were trypsinated 24 h after transfection. Cells and quanta from each

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3 The abbreviations used are: PMΦ, peritoneal macrophage(s); BMMΦ, bone marrow-derived macrophage(s); MEF, mouse embryonic fibroblasts; qPCR, quantitative PCR; qRT-PCR, quantitative RT-PCR; H3K4me3, trimethylation of histone H3 at lysine 4.
transfection were reseeded in four separate culture vessels for differential treatments. To induce ectopic IkBζ expression, Raw264.7/TetOn-IkBζ cells were cultured in the presence of 2 μg/ml doxycycline. After additional 16 h, doxycycline-treated and untreated cells were incubated with 1 μg/ml LPS. Cells were harvested 48 h after transfection, and luciferase assays were performed with the Dual-Luciferase reporter assay system (Promega Corp., Madison, WI).

Quantitative RT-PCR—Whole cell RNA was isolated using the RNeasy mini kit (Qiagen, Hilden, Germany) and reverse-transcribed (QuantiTect kit, Qiagen) according to the instructions of the manufacturer. Quantitative PCR (qPCR; LightCycler 480 II, Roche) was performed using SYBR Green/ROX qPCR Master Mix (Fermentas, Sankt Leon-Rot, Germany) as described in the two-step cycling protocol of the manufacturer (96-well plates, 20 μl reaction). The following primer pairs were used: Cxcl9, 5′-GAT TTT TAC TAA TTT AGT GTC TTG C-3′ and 5′-GGA ACC ATA GTG ATG AGG AAC GC-3′; Gapdh, 5′-ACC ACA GTC CAT GCC ATC AC-3′ and 5′-CAC CAC CCT GTT GCT GTA GCC-3′; Gbp4, 5′-ATG GTC ATT CCC TTG TGG AAA G-3′ and 5′-AAG GAG TGA TAA AAC GCT GCT C-3′; Elam1, 5′-CTC ACT CCT GAC ATC GTC-3′ and 5′-ACG TTG TAA GAA GGC ACA TGG-3′; Il6, 5′-AGT TGC CTT CTT GGG ACT GA-3′ and 5′-TCC AGC ATT TCC CAG AGA AC-3′; Il10, 5′-AGC TT TCT GAA GAA GGC CCC T-3′ and 5′-GAG CTT GTA GAC ACC TGG TT-3′; Nfkbia, 5′-CTC ACT CCT GAC ATC GTC CTC-3′ and 5′-ACG TTG TAA GAA GGC ACA TGG-3′; Nfkbia, 5′-TAT CGG GTG ACA CAG TCG GA-3′ and 5′-TGA ATG GAC TTC CCC TTC AG-3′; Stat1, 5′-GCT GCC TAT GAT GTC TCG TTT-3′ and 5′-TGC TTT TCC GTA TGT GTT GCT-3′; and Tnfα, 5′-CTT CAG CCT CTT TTC CTG CTT C-3′ and 5′-GTT GTG GGT GAG GAC CA-3′. Quantification of reverse-transcribed mRNA was performed using the second derivative maximum-based advanced relative quantification algorithm of the Roche LightCycler 480 software (V1.5).

Immunoblotting—Immunoblotting was performed as described previously (30) using anti-β-actin (A2228, Sigma-Aldrich, 1:10,000), rabbit anti-mouse Stat1 (9172, Cell Signaling Technology, Frankfurt, Germany, 1:2000), rabbit anti-mouse pStat1 (Tyr(P)701, clone 58D6, Cell Signaling Technology, 1:2000), and rabbit anti-IκBζ, which was produced as described previously (15).

Chromatin Immunoprecipitation—ChIP experiments were performed with the HighCell ChIP kit (Diagenode, Seraing, Belgium) using anti-histone H3 (trimethyl-Lys4) antibodies (Bioz Antikörper, Aachen, Germany). ChIP efficiencies were determined by qPCR on a LightCycler 480 II (Roche) using Maxima Hot Start TaqDNA polymerase according to the two-step cycling protocol of the manufacturer (96-well plates, 20 μl volume). The following primer pairs spanning κB-binding sites of the murine Il6, Il10, and Tnfα gene promoters were used: Il6, 5′-CGA TGG TAA ACG ACG TCA TAT GTC GCA-3′ and 5′-CTC CAG AGC AGA ATG AGC TAC AGA CAT C-3′; Il10, 5′-TAG AAG AGG GAG GAG GAG GAG CC-3′ and 5′-TGT GGC TTT GGT AGT GCA AG-3′; and Tnfα, 5′-CCG CAG ATT GCC ACA GAA TC-3′ and 5′-CCA GTG AGT GAA AGG GAC AG-3′. Primers covering portions of the Gapdh promoter (5′-GGG GTT GTC GTT TCA CTA CCG-3′ and 5′-CAG AGA CCT GAA TGC TGC TCC C-3′) and Actb promoter (5′-TGC ATA TCC ACG TGA CAT CCA-3′ and 5′-GCA GCA TTT TTT TAC CTC CTC-3′) served as controls. The specificities of the primers were verified, and PCR efficiencies were determined. Samples were analyzed according to the instructions of the manufacturer. Relative promoter occupancies were calculated as described previously (15).

Measurement of Cytokine Concentrations—Concentrations of cytokines in cell culture supernatants were measured using the mouse cytokronic bead array system (mouse anti-II-6, anti-II-10, and anti-TNFα) according to the instructions of the manufacturer (BD Biosciences). Before stimulation of cells in 96-well plates, the culture medium was exchanged with 200 μl of fresh medium per well. In the case of BM-MΦ, cytokine concentrations in culture supernatants were directly compared and expressed as cytokine amounts per volume. To avoid mouse-specific differences in peritoneal cell counts, cytokine concentrations in supernatants of PMB were normalized to the protein content of cell lysates. To this end, 50 μl of 0.2 M NaOH was added to each well after aspiration of the supernatants, and protein concentrations in lysates were measured with the BCA protein assay (Thermo Scientific, Bonn, Germany).

Statistical Analysis—Values are expressed as mean ± S.D. or S.E. for the indicated numbers of independent experiments. For statistical comparisons, hypotheses were tested using an unpaired Student’s t test.

Results

IkBζ Is Essential for Il10 Expression in Mouse Embryonic Fibroblasts and Peritoneal Macrophages—Although IkBζ transcriptionally induces several pro-inflammatory genes in cells of the monocyte lineage, Nfkbia−/− mice exhibit features of chronic inflammation. To explore this discrepancy, we focused our analyses on IL-10 as one of the major anti-inflammatory mediators. Initially, we examined the gene expression profile of wild-type and Nfkbia−/− MEFs by quantitative RT-PCR (qRT-PCR). As expected, LPS-challenged Nfkbia−/− MEFs showed reduced expression of the IkBζ-dependent target gene Il6 (16) compared with wild-type MEFs (Fig. 1A). In contrast to Il6, expression of Tnfα, an IkBζ-independent NF-κB target gene (15, 16), was readily induced by LPS in wild-type and even more strongly in Nfkbia−/− MEFs. Intriguingly, analysis of Il10 expression revealed strongly diminished transcript levels in Nfkbia−/− MEFs compared with wild-type MEFs (Fig. 1A).

Because IL-10 is predominantly expressed by macrophages, we performed additional experiments in PMΦ from wild-type and Nfkbia−/− mice. We initially assayed the expression of macrophage surface markers, such as F4/80, CD11b, CD11c, and Ly6G/C (supplemental Fig. 1), and classical macrophage functions, such as phagocytosis, migration, and oxidative burst (supplemental Fig. 2). These analyses did not reveal significant differences between the two genotypes, indicating that PMΦ are a suitable experimental system. Because IFNγ-primed PMΦ have been used previously in studies on IkBζ-dependent gene regulation (15), we next analyzed the gene expression profiles of naïve and IFNγ-primed wild-type and Nfkbia−/− PMΦ. In line with published data (15), expression of Il6 was only slightly
Different in naïve PM from both genotypes but strongly reduced in IFN-β-primed PM from Nfkbiz−/− mice compared with wild-type cells. The expression levels of Tnfa did not significantly differ in wild-type and Nfkbiz−/− PM (Fig. 1B). Importantly, compared with wild-type PM, both naïve and IFN-γ-primed PM from Nfkbiz−/− mice revealed strongly reduced mRNA levels of Il10 (Fig. 1C).

IkBζ Deficiency Reduces IL-10 Secretion in Bone Marrow Macrophages—Having shown that Il10 expression is diminished in Nfkbiz−/− MEFs and PM, we further analyzed IL-10 mRNA and protein expression in BMMΦ. Again, cells were left untreated (naïve) or pretreated with IFNγ before challenging with LPS. Gene expression analyses revealed comparable mRNA levels of Tnfa in naïve and IFN-γ-primed BMMΦ, whereas Il6 expression was significantly reduced in the absence of IkBζ (Fig. 2A and B). In line with the previous experiments, expression of Il10 mRNA was strongly reduced in Nfkbiz−/− BMMΦ compared with wild-type BMMΦ.

We further analyzed the kinetic of Il10 expression in BMMΦ of both genotypes. LPS stimulation of wild-type BMMΦ induced Il10 mRNA expression already after 4 h, with maximal expression 8 h after addition of LPS (Fig. 2C). This time-dependent expression is comparable with that of Il6 and Ccl2, which have been shown to be secondary response genes in the NF-κB signaling cascade. In line with the previous results, LPS-treated Nfkbiz−/− BMMΦ showed only marginally increased Il10 mRNA expression even 8 h after stimulation. In a similar setup, we analyzed cytokine concentrations in supernatants from LPS-challenged BMMΦ. The amount of TNFα detected in supernatants from LPS-challenged wild-type and Nfkbiz−/− BMMΦ was similar and served as a positive control (Fig. 2D). In line with the qRT-PCR analysis, the concentration of IL-10 increased in supernatants from LPS-challenged wild-type BMMΦ, reaching a maximum after 16 h, whereas, in supernatants from Nfkbiz−/− BMMΦ, only basal IL-10 levels were detected. TNFα levels were comparable in supernatants from BMMΦ of both genotypes (Fig. 2E), whereas the amount of IL-6 was significantly reduced in Nfkbiz−/− BMMΦ compared with wild-type cells (Fig. 2F). Thus, these data clearly indicate IkBζ-dependent regula-
tion of Il10 mRNA expression and protein secretion in macrophages.

**IkBζ-regulated IL-10 Expression Is Independent of Macrophage Polarization**—The classical activation of macrophages induces M1 polarization, whereas alternative activation results in M2 polarization. Previous gene expression analysis revealed that the regulation of Il6 gene expression depends on the macrophage polarization state (15). Hence, we wondered whether IL-10 expression is also influenced by the polarization state. Therefore, we quantified the concentration of cytokines in supernatants from LPS-challenged naïve and M1- and M2-polarized BMMΦ. As a positive control, we analyzed the secretion of TNFα, which was readily detectable in supernatants from naïve and classically (M1) and alternatively (M2) activated wild-type and Nfkbiz−/− BMMΦ (Fig. 3A). The concentrations of both IL-6 and IL-10 were significantly decreased in Nfkbiz−/− BMMΦ compared with wild-type BMMΦ in case of all three polarization states (Fig. 3, A–C). Thus,
expression of IL-10 is not affected by the macrophage polarization state.

**IkBζ Overexpression Induces Il10 Promoter Activity and Gene Expression**—Because our investigations so far relied on knock-out systems, we additionally employed the macrophage-like cell line Raw264.7, which was genetically modified to enable inducible doxycycline-dependent IkBζ expression. Treatment of the Raw264.7/TetOn-IkBζ cells with doxycycline resulted in the robust expression of IkBζ, which was further increased by stimulation with LPS (Fig. 4A).

We next investigated the impact of transgenic IkBζ expression on the induction of various IkBζ-independent and -dependent genes. Quantitative RT-PCR analyses confirmed a dose-dependent induction of Nfkbiz transcription in Raw264.7/TetOn-IkBζ by doxycycline (Fig. 4B). The expression of the IkBζ-independent NF-κB target genes Nfkbia and Elam1 remained unaffected by doxycycline, whereas expression of the IkBζ-dependent target genes Il6 and Lcn2 correlated with the concentration of doxycycline and the mRNA levels of Nfkbia. Similar to Il6 and Lcn2, expression of Il10 was enhanced in the presence of doxycycline and induced Nfkbia (Fig. 4B). Thus, expression of IkBζ is sufficient for induction of Il10 expression.

We further analyzed whether IkBζ directly activates the Il10 promoter. In a first set of experiments, we transfected Raw264.7/TetOn-IkBζ cells with Elam1, Lcn2, Il6, and Il10 reporter gene constructs and analyzed whether doxycycline-induced IkBζ expression results in increased luciferase activity. No reporter activity was induced from the empty vector backbone and the IkBζ-independent promoter of Elam1 (Fig. 4C). In contrast, IkBζ expression clearly induced luciferase activity from the Il6 and the Lcn2 promoters, which are both regulated by IkBζ (16, 31). Importantly, doxycycline-induced expression of IkBζ also strongly induced luciferase expression from the Il10 promoter construct (Fig. 4C).

**IkBζ Targets the Proximal Promoter Region of the Il10 Genomic Locus**—Analysis of the Il10 promoter reveals two NF-κB consensus sites that are located in distal (−1115 to −1106 bp) and proximal (−55 to −46 bp) promoter regions (Fig. 5A). Because these regions potentially serve as anchor points for IkBζ-mediated transcription, we used reporter constructs containing various truncated versions of the Il10 promoter. Upon transfection of Raw264.7 cells, LPS induced a strong activation of the full-length Il10 promoter (Fig. 5B). Truncations of the Il10 promoter and deletion of the distal...
NF-κB-binding site resulted only in a minor reduction of luciferase activity, indicating that the proximal NF-κB site is important for Il10 promoter activation.

The recruitment of IκBζ to promoter regions is dependent on the DNA-binding subunit p50. To verify that the proximal NF-κB-binding site is responsible for IκBζ-mediated Il10 induction, we co-transfected Raw264.7 cells with the reporter construct of the proximal Il10 promoter region (−158 to +64 bp) together with expression vectors for IκBζ and p50. The single transfection of Nfkbia did not result in luciferase activity (Fig. 5C). Furthermore, consistent with an inhibitory role of p50 homodimers (10), the sole expression of p50 even reduced reporter gene activity. However, upon co-transfection of IκBζ and p50, luciferase activity increased by 2- to 3-fold (Fig. 5C), indicating a direct activation of Il10 gene expression by the complex of IκBζ and p50.

Active gene transcription is associated with open chromatin and trimethylation of histone H3 at lysine 4 (H3K4me3) at promoter regions. We and others have previously shown that IκBζ is required for formation of the transcription preinitiation complex and H3K4 trimethylation at targeted loci (15, 19, 32). To further substantiate a role of the proximal Il10 promoter for IκBζ-mediated gene expression, we analyzed the degree of H3K4 trimethylation in the presence and absence of Nfkbia. To this end, we employed qPCR-coupled ChIP analysis of the endogenous proximal Il10, Il6, and Tnfa promoter regions using an H3K4me3-specific antibody. Compared with unstimulated wild-type BMMΦ, LPS stimulation resulted in an ~20-fold enrichment of H3K4 trimethylation at the proximal Il10 promoter (Fig. 5D). An even stronger enrichment was seen for the Il6 promoter, whereas H3K4 trimethylation of the proximal Tnfa promoter was barely affected. Importantly, compared with wild-type BMMΦ, no H3K4 trimethylation was detectable at the endogenous Il6 and Il10 promoters in IκBζ-deficient cells (Fig. 5D). The transcription-associated H3K4 trimethylation at the Il10 promoter was exclusively observed in the presence of IκBζ. Thus, we conclude that IκBζ regulates IL-10 expression by directly binding to the proximal region of the Il10 promoter together with p50.

IL-10 Partially Reverses the M1 Phenotype of Nfkbia−/− BMMΦ—Our previous gene expression analysis comparing IκBζ-proficient and -deficient macrophages (GEO accession no. GSE43075, Ref. 15) revealed a strong up-regulation of several M1 markers in the absence of IκBζ, which is consistent with
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**FIGURE 5. IκBζ regulates IL10 promoter activity.** A, schematic of the murine Il10 promoter. The dark boxes indicate the positions of κB binding sites. B, Raw264.7 cells were transfected with the empty luciferase reporter gene vector (ctrl) or reporter gene constructs harboring the indicated regions of the Il10 promoter. Luciferase activity was analyzed after 24 h of incubation in the absence or presence of LPS. Values are mean ± S.D. from three experiments. *, statistical significance comparing luciferase activity in the presence and absence of LPS. AU, arbitrary units. C, Raw264.7 cells were transfected with a luciferase reporter construct of a truncated Il10 promoter (−158 to +64 bp) harboring the proximal κB binding site together with pcDNA4 expression vectors for Nfkb1 (p50), IκBζ, or a combination thereof. Luciferase activity was analyzed 24 h after transfection. Promoter activity obtained after transfection of the empty pcDNA4 vector was set as 1. Values are mean ± S.D. of three experiments. D, chromatin from LPS-treated (5 h) naive wild-type and Nfkbid−/− BMMφ was subjected to ChIP assays applying an H3K4me3-specific antibody. The degree of H3K4 trimethylation of the Il10, Il6, and Tnfa promoters was determined via qPCR. ChIP analysis with an isotype control antibody served as a control. Values are mean ± S.D. from three experiments. *, statistical significance comparing wild-type and Nfkbid−/− cells.

The pro-inflammatory phenotype of Nfkbid−/− mice. Examples for the preferential M1 polarization are the elevated mRNA expression of Stat1, Gbp4, and Cxcl9 as well as the increased expression and phosphorylation of Stat1 protein in LPS-stimulated Nfkbid−/− BMMφ compared with the wild-type counterparts (Fig. 6, A and B).

To investigate a potential role of IL-10 in this M1 polarization, we incubated Nfkbid−/− BMMφ in the presence of LPS and varying concentrations of IL-10 and examined the expression of the M1 markers Stat1 and Gbp4. Quantitative RT-PCR revealed a dose-dependent reduction of Stat1 and Gbp4 expression by exogenous IL-10 (Fig. 6C), whereas Cxcl9 expression remained largely unaffected (data not shown). Importantly, when Nfkbid−/− BMMφ were incubated with IL-10-deficient supernatants from LPS-treated wild-type BMMφ, a significant reduction of Stat1 and Gbp4 expression was also detected (Fig. 6D). The reduction of Gbp4 expression roughly corresponded to the reduction observed with 2 ng/ml IL-10, a concentration similar to that present in supernatants of LPS-stimulated BMMφ (Fig. 3A). Thus, these results not only show that IL-10 signaling is functional in Nfkbid−/− BMMφ but, moreover, indicate that the lack of IL-10 in Nfkbid−/− BMMφ at least partially contributes to their enhanced M1 polarization.

**Discussion**

Growing evidence suggests that the induction of NF-κB-regulated genes is not solely defined by the nuclear translocation of NF-κB but that different NF-κB target genes have individual expression profiles regarding kinetic, stimulus, or cell type, thereby ensuring a selectivity of an immune response. Several recent studies identified a subfamily of atypical IκB proteins as important “specifiers” that select particular κB-sites to be activated or repressed under certain conditions (14).

IκBζ is mostly regarded as a pro-inflammatory regulator, as demonstrated e.g. by its requirement for Th17 differentiation and expression of particular pro-inflammatory cytokines. Nevertheless, Nfkbid−/− mice show a pro-inflammatory phenotype and M1 hyperpolarization of macrophages (15, 16, 24, 25), suggesting that so far unknown anti-inflammatory mediators might be controlled by IκBζ. In this study, we found that induction of the potent anti-inflammatory cytokine IL-10 by LPS but also by TLR2 agonists (data not shown) was strictly dependent on IκBζ and strongly reduced in Nfkbid−/− mice. These results were supported by the finding that the doxycycline-inducible expression of ectopic IκBζ in Raw264.7 macrophages tightly correlated with increased Il10 mRNA levels. Moreover, reporter analysis revealed that the proximal κB site of the Il10 promoter was responsible for IκBζ-mediated Il10 expression. The recruitment of IκBζ was associated with histone H3K4 trimethylation of the proximal promoter region as a marker of active gene transcription. Interestingly, in the absence of IκBζ, H3K4 trimethylation did not occur, which, along with other lines of evidence, suggests that chromatin remodeling is essential for IκBζ action. Thus, our results in knockout and overexpression models suggest that transcriptional regulation of Il10 directly depends on IκBζ.

So far, the expression of IL-10 in macrophages is known to be primarily regulated by transcription factors such as SP1, C/EBPβ, IRF1, and STAT3 (28, 33–35), whereas a role of different NF-κB proteins is relatively unknown. Because of the lack of a transcription activation domain, p50 NF-κB homodimers, which retain their ability to bind to κB sites, are thought to be transcriptional repressors. Interestingly, although not investigating atypical IκB proteins, earlier studies already showed that p50 homodimers bind to the proximal Il10 promoter and activate Il10 transcription in primary macrophages (36).

Moreover, in contrast to IκBζ, Bcl-3, a related atypical IκB protein that also requires p50 for co-regulation, negatively regulates Il10 transcription in macrophages (27, 37), although the exact role of Bcl-3 for Il10 expression is controversial (38). Bcl3 knockout mice show enhanced susceptibility to infection with *Listeria monocytogenes*, which is due to enhanced expression of
IL-10, resulting in diminished levels of IL-12p70 and IFNγ. These results suggest that atypical IκB proteins, such as Bcl-3 and IκBζ, might regulate gene expression in an opposite manner, which is also underlined by the fact that IL-12p70 and IFNγ are direct IκBζ targets. Likewise, Bcl-3 and IκBζ have an antagonistic effect on CCL2 expression in macrophages. Although Bcl-3 inhibits the expression of CCL2, IκBζ promotes the expression of this chemokine (15, 38). Another example of such opposite gene regulation by atypical IκB proteins concerns IκBζ and IκBNS. For instance, although IκBζ is required for IL-6, IL-12p40, and G-CSF expression (15, 16), IκBNS apparently inhibits transcription of these cytokines (39, 40). Interestingly, our gene expression profiling suggest that atypical IκB proteins might also influence each other at the transcriptional level and, moreover, compete with each other for p50-mediated DNA binding. Together, these findings suggest that atypical IκB proteins form a complex network in controlling NF-κB responses.

In addition to transcription factor binding, previous studies suggested that II10 expression is regulated by changes in the chromatin structure at the IL10 locus. The histone deacetylase HDAC11 has been found to inhibit IL-10 expression (41), whereas phosphorylation of histone H3 at serine 10 is needed for transcriptional activation of the II10 promoter (42). A recent study found that IκBζ recruits the epigenetic modifier Tet2 to selective promoter regions independent of DNA meth-
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ylation (43). IkBζ further mediates chromatin remodeling by recruiting the SWI/SNF complex to target genes, thereby enhancing promoter accessibility (32). The same mechanism presumably underlies the regulation of Il10 expression because we found that Il10 promoter accessibility and H3K4 trimethylation were reduced in Nfkbia−/− cells. Thus, it will be interesting to explore whether Bcl-3 and IkBζ mediate their antagonistic effects at the Il10 promoter by recruiting distinct histone-modifying enzymes.

Although cells of the macrophage lineage are a major source of IL-10, several other cell types of the innate and adaptive immune system can express this cytokine (6, 7). Further studies are needed to explore whether the strict control of IL-10 expression by IkBζ is also relevant to other cell types. Our exemplary investigation of wild-type and Nfkbia−/− MEFs indicates that the described mechanism is not restricted to macrophages. Interestingly, previous gene expression analysis revealed that IkBζ-deficient macrophages show a bias toward M1 polarization, evidenced by the increased expression of certain M1 markers (15). It is worth mentioning that p50-deficient mice also show exacerbated M1-driven inflammation and reduced M2 polarization of their macrophages (44), although several phenotypic alterations are distinct between Nfkbia−/− and p50-deficient mice.

In functional studies, we found that the increased mRNA expression of Stat1 and Gpb4 could be partially reverted not only by IL-10 supplementation but also by IL-10-proficient supernatants from wild-type macrophages even though no reduction in Cxcl9 expression was observed. It was not the intention of our study to investigate the role of IkBζ-mediated IL-10 expression in macrophage polarization. Our results, however, indicate that decreased IL-10 expression contributes to at least some of the features of M1 polarization in Nfkbia−/− mice. In line, IL-10-producing monocytes have been found to preferentially differentiate to M2 macrophages (45, 46).

Dysregulation of Il10 expression has been linked to several immune disorders. Transgenic mice overexpressing IL-10 in macrophages exhibit increased susceptibility to bacterial infections and septic shock (47). Excessive IL-10 production has also been linked to impaired tumor immune surveillance (48, 49). In contrast, the absence of IL-10 results in spontaneous inflammatory bowel disease (8), emphasizing its protective role in inflammatory and autoimmune conditions. We did not detect spontaneous colitis in Nfkbia−/− mice, which might be caused by the genetic background because intestinal lesions have been reported to be least severe in C57BL/6 mice (50). It is, however, worth mentioning that Nfkbia−/− mice exhibit an increased susceptibility to dextran sodium sulfate-induced colitis.

In summary, we have uncovered an essential novel regulatory mechanism of Il10 gene regulation in macrophages. We demonstrate that IkBζ through p50-mediated recruitment to the proximal Il10 promoter and subsequent histone H3 modification, enables transcription of the Il10 locus. Because IL-10 plays a beneficial role in several inflammatory diseases, Nfkbia−/− mice are an interesting model system for evaluating IkBζ as a potential therapeutic target in inflammatory diseases.

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**References**

1. Gordon, S., and Taylor, P. R. (2005) Monocyte and macrophage heterogeneity. Nat. Rev. Immunol. 5, 953–964
2. Ginhoux, F., and Jung, S. (2014) Monocytes and macrophages: developmental pathways and tissue homeostasis. Nat. Rev. Immunol. 14, 392–404
3. Laskin, D. L., Sunil, V. R., Gardner, C. R., and Laskin, J. D. (2011) Macrophages and tissue injury: agents of defense or destruction? Annu. Rev. Pharmacol. Toxicol. 51, 267–288
4. Murray, P. J., Allen, J. E., Biswas, S. K., Fisher, E. A., Gilroy, D. W., Goerd, S., Gordon, S., Hamilton, J. A., Ishikawa, L. B., Lawrence, T., Locati, M., Mantovani, A., Martinez, F. O., Mege, J. L., Mosser, D. M., et al. (2014) Macrophage activation and polarization: nomenclature and experimental guidelines. Immunity 41, 14–20
5. Sica, A., and Mantovani, A. (2012) Macrophage plasticity and polarization: in vivo veritas. J. Clin. Invest. 122, 787–795
6. Moore, K. W., de Waal Malefyt, R., Coffman, R. L., and O’Garra, A.(2001) Interleukin-10 and the interleukin-10 receptor. Annu. Rev. Immunol. 19, 683–765
7. Saravia, M., and O’Garra, A.(2010) The regulation of IL-10 production by immune cells. Nat. Rev. Immunol. 10, 170–181
8. Kühn, R., Löhler, J., Rennick, D., Rajewsky, K., and Müller, W.(1993) Interleukin-10-deficient mice develop chronic enterocolitis. Cell 75, 263–274
9. O’Garra, A., Vieira, P. L., Vieira, P., and Goldfeld, A. E.(2004) IL-10-producing and naturally occurring CD4+ Treg: limiting collateral damage. J. Clin. Invest. 114, 1372–1378
10. Ockinghaus, A., Hayden, M. S., and Ghosh, S.(2011) Crosstalk in NF-κB signaling pathways. Nat. Immunol. 12, 695–708
11. Hinz, M., Arslan, S. Ç., and Scheidereit, C.(2012) It takes two to tango: NF-κB family, human p50-deficient mice. J. Mol. Biol. 424, 4761–4767
12. Smale, S. T. (2011) Hierarchies of NF-κB target-gene regulation. Nat. Immunol. 12, 689–694
13. Smale, S. T.(2010) Selective transcription in response to an inflammatory stimulus. Cell 140, 833–844
14. Schuster, M., Annemann, M., Plaza-Sirvent, C., and Schmitz, I.(2013) Atypical IkBζ proteins: nuclear modulators of NF-κB signaling. Cell Commun. Signal. 11, 23
15. Hildebrand, D. G., Alexander, E., Hörber, S., Lehn, S., Obermayer, K., Münck, N. A., Rothfuss, O., Frick, J. S., Morimatsu, M., Schmitz, I., Roth, J., Ehrchen, J. M., Essmann, F., and Schulze-Osthoff, K.(2013) IkBζ is a transcriptional key regulator of CCL2/ MCP-1. J. Immunol. 190, 4812–4820
16. Yamamoto, M., Yamazaki, S., Uematsu, S., Sato, S., Hemmi, H., Hoshiba, K., Kaiho, T., Kuswara, H., Takeuchi, O., Takeda, K., Saitoh, T., Yamaoka, S., Yamamoto, N., Yamamoto, S., Mutu, T., et al. (2004) Regulation of Toll/IL-1 receptor-mediated gene expression by the inducible nuclear protein IkBζ. Nature 430, 218–222
17. Totzek, G., Essmann, F., Pohlmann, S., Lindenblatt, C., Jánicek, R. U., and Schulze-Osthoff, K.(2006) A novel member of the IκB family, human IκBζ, inhibits transactivation of p65 and its DNA binding. J. Biol. Chem. 281, 12645–12654
18. Trinh, D. V., Zhu, N., Farhang, G., Kim, B. J., and Huxford, T.(2008) The nuclear IkBζ protein IκBζ specifically binds NF-κB p50 homodimers and forms a ternary complex on κB DNA. J. Mol. Biol. 379, 122–135
19. Kayama, H., Ramirez-Carrozzi, V. R., Yamamoto, M., Mizutani, T., Ku-
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homodimers differentially regulate pro- and anti-inflammatory cytokines in macrophages. J. Biol. Chem. 281, 29601–29605

37. Wessells, J., Baer, M., Young, H. A., Claudio, E., Brown, K., Siebenlist, U., and Johnson, P. F. (2004) BCL-3 and NF-κB p50 attenuate lipopolysaccharide-induced inflammatory responses in macrophages. J. Biol. Chem. 279, 49995–50003

38. Carney, R. J., Ruan, Q., Palmer, S., Hillard, B., and Chen, Y. H. (2007) Negative regulation of toll-like receptor signaling by NF-κB p50 ubiquitination blockade. Science 317, 657–658

39. Hirota, T., Lee, P. Y., Kuwata, H., Yamamoto, M., Matsumoto, M., Kaewse, I., Akira, S., and Takeda, K. (2005) The nuclear IkBζ protein IkBζNS selectively inhibits lipopolysaccharide-induced IL-6 production in macrophages of the colonic lamina propria. J. Immunol. 174, 3650–3657

40. Kuwata, H., Matsumoto, M., Atarashi, K., Morishita, H., Hirota, T., Koga, R., and Takeda, K. (2006) IkBNS inhibits induction of a subset of Toll-like receptor-dependent genes and limits inflammation. Immunity 24, 41–51

41. Villagra, A., Cheng, F., Wang, H. W., Suarez, I., Glezak, M., Maurin, M., Nguyen, D., Wright, K. L., Atadja, P. W., Bhalla, K., Pinilla-Ibarz, J., Seto, E., and Sotomayor, E. M. (2009) The histone deacetylase HDAC11 regulates the expression of interleukin 10 and immune tolerance. Nat. Immunol. 10, 92–100

42. Lucas, M., Zhang, Y., Prasanna, V., and Mosser, D. M. (2005) ERK activation following macrophage FcγR ligation leads to chromatin modifications at the IL-10 locus. J. Immunol. 175, 469–477

43. Zhang, Q., Zhao, K., Shen, Q., Han, Y., Gu, Y., Li, X., Zhao, D., Liu, Y., Wang, C., Zhang, X., Su, X., Liu, J., Ge, W., Levine, R. L., Li, N., and Cao, X. (2015) Tet2 is required to resolve inflammation by recruiting Hdac2 to specifically repress IL-6. Nature 525, 389–393

44. Porta, C., Rimoldi, M., Raes, G., Brey, L., Ghezzi, P., Di Libertato, D., Dieli, F., Ghisletti, S., Natoli, G., De Baetselier, P., Mantovani, A., and Sica, A. (2009) Tolerance and M2 (alternative) macrophage polarization are related processes orchestrated by p50 nuclear factor κB. Proc. Natl. Acad. Sci. U.S.A. 106, 14978–14983

45. Prasse, A., Germann, M., Pechkovsky, D. V., Markert, A., Verres, T., Stahl, M., Melchert, I., Luttman, W., Müller-Quernheim, J., and Zissel, G. (2007) IL-10-producing monocytes differentiate to alternatively activated macrophages and are increased in atopic patients. J. Allergy Clin. Immunol. 119, 464–471

46. Makita, N., Hizukuri, Y., Yamashiro, K., Murakawa, M., and Hayashi, Y. (2015) IL-10 enhances the phenotype of M2 macrophages induced by IL-4 and confers the ability to increase eicosanoid migration. Int. Immunol. 27, 131–141

47. Lang, R., Rutschman, R. L., Greaves, D. R., and Murray, P. J. (2002) Autoimmune activation of macrophages in transgenic mice constitutively overexpressing IL-12 under control of the human CD68 promoter. J. Immunol. 168, 3402–3411

48. Béguelin, W., Sawh, S., Chambwe, N., Chan, F. C., Jiang, Y., Cho, J. W., Scott, D. W., Chalmers, A., Geng, H., Tsikitas, L., Tam, W., Bhagat, G., Gascony, R. D., and Shakhovnik, R. (2015) IL10 receptor is a novel therapeutic target in DLBCLs. Leukemia 29, 1684–1694

49. Ruffell, B., Chang-Strachan, D., Chan, V., Rosenbusch, A., Ho, C. M., Pryer, N., Daniel, D., Hwang, E. S., Rugo, H. S., and Couchman, L. M. (2014) Macrophage IL-10 blocks CD8+ T cell-dependent responses to chemotherapy by suppressing IL-12 expression in intratumoral dendritic cells. Cancer Cell 26, 623–637

50. Berg, D. J., Davidson, N., Kühn, R., Müller, W., Menon, S., Holland, G., Thompson-Snipes, L., Leach, M. W., and Rennick, D. (1996) Enterocolitis and colon cancer in interleukin-10-deficient mice are associated with aberrant cytokine production and CD4 Th1-like responses. J. Clin. Invest. 98, 1010–1020

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