THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 291, NO. 24, pp. 12851–12861, June 10, 2016

The Atypical Inhibitor of NF-κB, IκBζ, Controls Macrophage Interleukin-10 Expression*[S]

Received for publication, January 30, 2016, and in revised form, April 11, 2016 Published, JBC Papers in Press, April 18, 2016, DOI 10.1074/jbc.M116.718825

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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.

Macrophages constitute a heterogeneous group of phagocytes that fulfill pro- as well as anti-inflammatory responses (1, 2). A pro-inflammatory response of innate immune cells is indispensable for host defense. However, dysregulation of innate immunity can result in severe damage of the affected host. Weak immune responses lead to prolonged infection and persistence of pathogens, whereas overshooting responses promote chronic inflammation and autoimmune disease (3). Therefore, tight control of defense mechanisms is essential for host protection against self-destructive, excessive, and undue 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 NF-κB 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 NF-κB subunits can be divided into two subgroups. RelA (p65), RelB, and c-Rel possess a C-terminal transcription activation domain, whereas p50 (Nfkb1) and p52 (Nfkb2) lack a transcription activation domain. Based on these structural differences, NF-κB 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 NF-κB signaling pathway and a great diversity of target genes is regulated by NF-κB, a precise control of NF-κB activity is required to avoid misguided immune responses. In fact, NF-κB 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, NF-κB 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

* This work was supported by Deutsche Forschungsgemeinschaft Grants SFB685 and GRK1302 and Bundesminsterium für Bildung und Forschung Grant AID-NET. The authors declare that they have no conflicts of interest with the contents of this article.

[S] This article contains supplemental Figs. 1 and 2.

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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 are so-called atypical IκB proteins, including Bcl-3, $I\kappa B_{NS}$, IκBζ, and IκBη (11, 14). Atypical IκBs differ markedly from classical cytosolic IκBs because they are mostly inducibly expressed and localized in the nucleus. Moreover, atypical IκBs do not exclusively act as inhibitors but can also activate the expression of secondary response genes.

The atypical IκB protein IκBζ has been recently implicated in differential NF-κB target gene expression in macrophages (15, 16) even though its physiological function remains largely unknown. The IκBζ-encoding *Nfkbiz* gene is rapidly induced as a primary NF-κB response gene by various inflammatory stimuli and, through association with the NF-κB subunit p50, is thought to exert its transcription-enhancing activity on secondary response genes mainly at the level of chromatin remodeling (12, 17, 18).

Recently, we showed that in macrophages, expression of CCL2 strictly depends on the presence of IκBζ and, consequently, that expression of CCL2 is abolished in macrophages from IκBζ-deficient mice (15). Furthermore, several other pro-inflammatory gene products, including IL-6, IL12p40, IL-17, IFNγ, and GM-CSF, have been found to be regulated by IκBζ (16, 19–23). Intriguingly, however, despite impaired expression of these pro-inflammatory cytokines, $Nfkbiz^{-/-}$ mice display a pro-inflammatory phenotype characterized by periocular inflammation, inflammatory skin alterations, and an M1 hyperpolarized macrophage state (15, 16, 24, 25). In view of the phenotype of $Nfkbiz^{-/-}$ mice, we therefore investigated a potential role of IκBζ for the regulation of IL-10 as an essential anti-inflammatory cytokine. Interestingly, although expression of *Il10* has been found previously to be inhibited by the atypical IκB protein Bcl-3 (26, 27), our results establish IκBζ as a novel and essential transcriptional inducer of *Il10* in macrophages. Our results therefore show for the first time that IκBζ is not only a pro-inflammatory mediator but also controls the activation of anti-inflammatory gene products.

Experimental Procedures

Animals—$Nfkbiz^{-/-}$ and control C57BL/6 mice were used at 6–8 weeks of age as described previously (23). $Nfkbiz^{-/-}$ 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 CO_2 asphyxiation. For isolation of peritoneal macrophages (PMΦ),[3] 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% CO_2, 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 (3×10^6 cells/ml), and cultured under low-oxygen conditions (5% CO_2, 5% O_2). After 7 days of differentiation, cells were washed with PBS, scraped off, and cultured at a density of 2×10^5 cells/cm^2 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-IκBζ 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-IκBζ cells were described previously (15) and cultured in the presence of 1 mg/ml neomycin (G418, Lonza). Cells were seeded at a density of 10^5 cells/cm^2 24 h before the experiment. Optionally, 2 μg/ml doxycycline (Sigma-Aldrich) was added to promote ectopic IκBζ expression. For pro-inflammatory stimulation, cells were treated with 1 μg/ml LPS.

Transfections and Reporter Gene Assays—Raw264.7 or Raw264.7/TetOn-IκBζ 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 IκBζ have been described previously (15, 17). For reporter gene assays, the following luciferase promoter constructs were used: pGL2basic-*Il10* and truncation mutants (28), pGL3basic-*Lcn2* (29), and pGL3basic-*Il6* and pGL3basic-*Elam1* (16). Cells were trypsinized 24 h after transfection. Cells and quanta from each

[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; H3K4me$_3$, trimethylation of histone H3 at lysine 4.

transfection were reseeded in four separate culture vessels for differential treatments. To induce ectopic IκBζ expression, Raw264.7/TetOn-IκBζ 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 (384-well plates, 10-μl reaction). The following primer pairs were used: *Cxcl9*, 5′-GAT TTG TAG TGG ATC GTG CCT C-3′ and 5′-GGA ACC CTA GTG ATA AGG AAT 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 GTG ATT CCC TTG TGG AAA G-3′ and 5′-AAG GAG TGA TAA AAC GCT GCT T-3′; *Elam1*, 5′-CTC ACT CCT GAC ATC GTC CTC-3′ and 5′-ACG TTG TAA GAA GGC ACA TGG-3′; *Il6*, 5′-AGT TGC CTT CTT GGG ACT GA-3′ and 5′-TCC ACG ATT TCC CAG AGA AC-3′; *Il10*, 5′-AGC CTT ATC GGA AAT GAT CCA GT-3′ and 5′-GGC CTT GTA GAC ACC TTG GT-3′; *Nfkbia*, 5′-CTC ACT CCT GAC ATC GTC CTC-3′ and 5′-ACG TTG TAA GAA GGC ACA TGG-3′; *Nfkbiz*, 5′-TAT CGG GTG ACA CAG TTG 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 TGT GCT-3′; and *Tnfa*, 5′-CCT CAG CCT CTT CTC CTT CCT-3′ and 5′-GGT GTG GGT GAG GAG CA-3′. Quantification of reverse-transcribed mRNA was performed using the second derivate 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-Lys^{4}) antibodies (Bioss 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 *Tnfa* gene promoters were used: *Il6*, 5′-CGA TGC TAA ACG ACG TCA CAT TGT GCA-3′ and 5′-CTC CAG AGC AGA ATG AGC TAC AGA CAT-3′; *Il10*, 5′-TAG AAG AGG GAG GAG GAG CC-3′ and 5′-TGT GGC TTT GGT AGT GCA AG-3′; and *Tnfa*, 5′-CCC 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 GCT GTG TCA CTA CCG-3′ and 5′-CAG AGA CCT GAA TGC TGC TTC C-3′) and *Actb* promoter (5′-TCG ATA TCC ACG TGA CAT CCA-3′ and 5′-GCA GCA TTT TTT TAC CCC 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 cytometric bead array system (mouse anti-IL-6, anti-IL-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 BMMΦ, 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 PMΦ 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

IκBζ Is Essential for Il10 Expression in Mouse Embryonic Fibroblasts and Peritoneal Macrophages—Although IκBζ transcriptionally induces several pro-inflammatory genes in cells of the monocyte lineage, *Nfkbiz*$^{-/-}$ 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 *Nfkbiz*$^{-/-}$ MEFs by quantitative RT-PCR (qRT-PCR). As expected, LPS-challenged *Nfkbiz*$^{-/-}$ MEFs showed reduced expression of the IκBζ-dependent target gene *Il6* (16) compared with wild-type MEFs (Fig. 1*A*). In contrast to *Il6*, expression of *Tnfa*, an IκBζ-independent NF-κB target gene (15, 16), was readily induced by LPS in wild-type and even more strongly in *Nfkbiz*$^{-/-}$ MEFs. Intriguingly, analysis of *Il10* expression revealed strongly diminished transcript levels in *Nfkbiz*$^{-/-}$ MEFs compared with wild-type MEFs (Fig. 1*A*).

Because IL-10 is predominantly expressed by macrophages, we performed additional experiments in PMΦ from wild-type and *Nfkbiz*$^{-/-}$ 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 IκBζ-dependent gene regulation (15), we next analyzed the gene expression profiles of naïve and IFNγ-primed wild-type and *Nfkbiz*$^{-/-}$ PMΦ. In line with published data (15), expression of *Il6* was only slightly

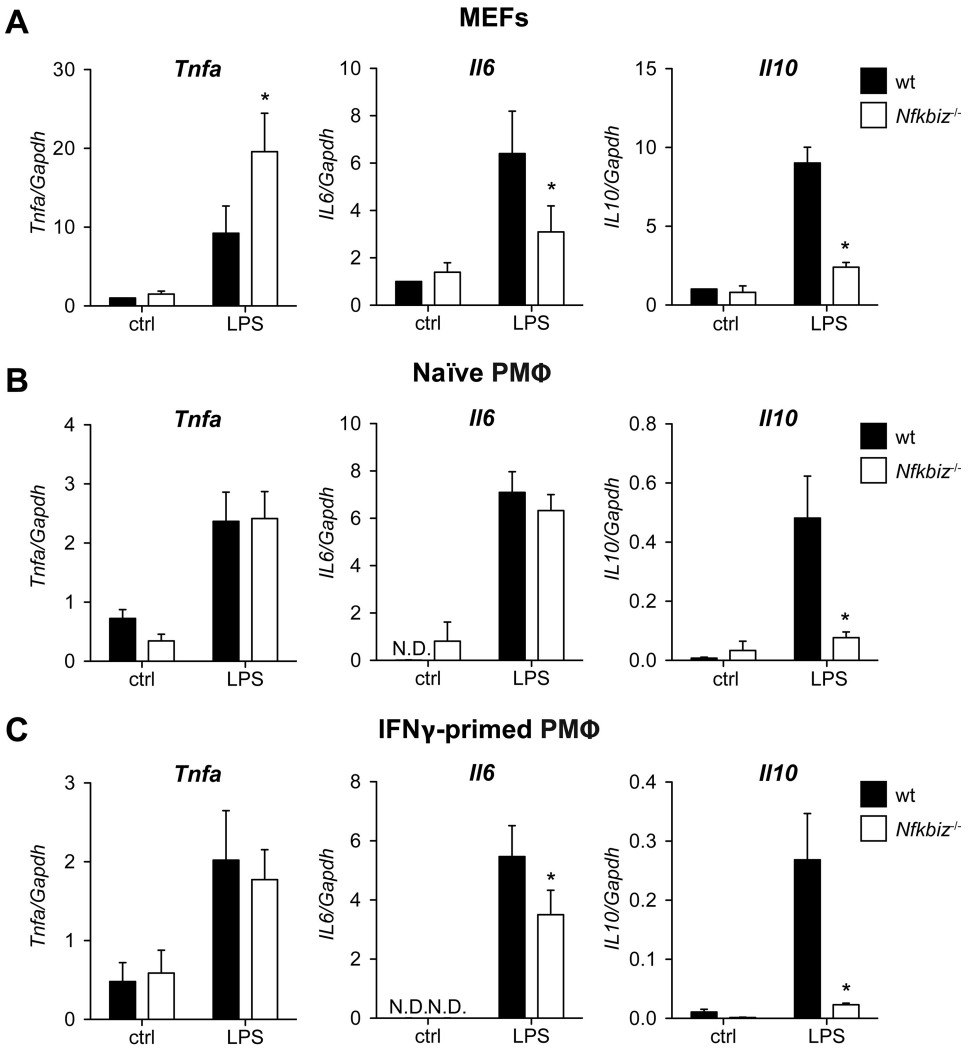

FIGURE 1. **IκBζ is essential for *Il10* expression in mouse embryonic fibroblasts and peritoneal macrophages**. *A*, MEFs were cultured in the presence of 1 μg/ml LPS for 5 h. Total RNA was isolated and subjected to qRT-PCR analysis to determine the expression levels of *Tnfa*, *Il6*, and *Il10*. Values are mean ± S.E. of four experiments. *ctrl*, control. *B* and *C*, naïve PMΦ (*B*) and PMΦ (*C*), classically primed with IFNγ, from wild-type and *Nfkbiz*$^{-/-}$ mice were either left untreated (*ctrl*) or cultured in the presence of 1 μg/ml LPS for 24 h before expression of *Tnfa*, *Il6*, and *Il10* was analyzed by qRT-PCR. Values were normalized to *Gapdh* and are mean ± S.E. of four experiments. *, statistical significance comparing wild-type and *Nfkbiz*$^{-/-}$ cells; *N.D.*, not detectable.

different in naïve PMΦ from both genotypes but strongly reduced in IFNγ-primed PMΦ from *Nfkbiz*$^{-/-}$ mice compared with wild-type cells. The expression levels of *Tnfa* did not significantly differ in wild-type and *Nfkbiz*$^{-/-}$ PMΦ (Fig. 1*B*). Importantly, compared with wild-type PMΦ, both naïve and IFNγ-primed PMΦ from *Nfkbiz*$^{-/-}$ mice revealed strongly reduced mRNA levels of *Il10* (Fig. 1*C*).

IκBζ Deficiency Reduces IL-10 Secretion in Bone Marrow Macrophages—Having shown that *Il10* expression is diminished in *Nfkbiz*$^{-/-}$ MEFs and PMΦ, 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 IFNγ-primed BMMΦ, whereas *IL6* expression was significantly reduced in the absence of IκBζ (Fig. 2, *A* 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. 2*C*). This time-dependent expression is comparable with that of *Il6* and *Ccl2* (15), 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. 2*D*). 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. 2*E*), whereas the amount of IL-6 was significantly reduced in *Nfkbiz*$^{-/-}$ BMMΦ compared with wild-type cells (Fig. 2*F*). Thus, these data clearly indicate IκBζ-dependent regula-

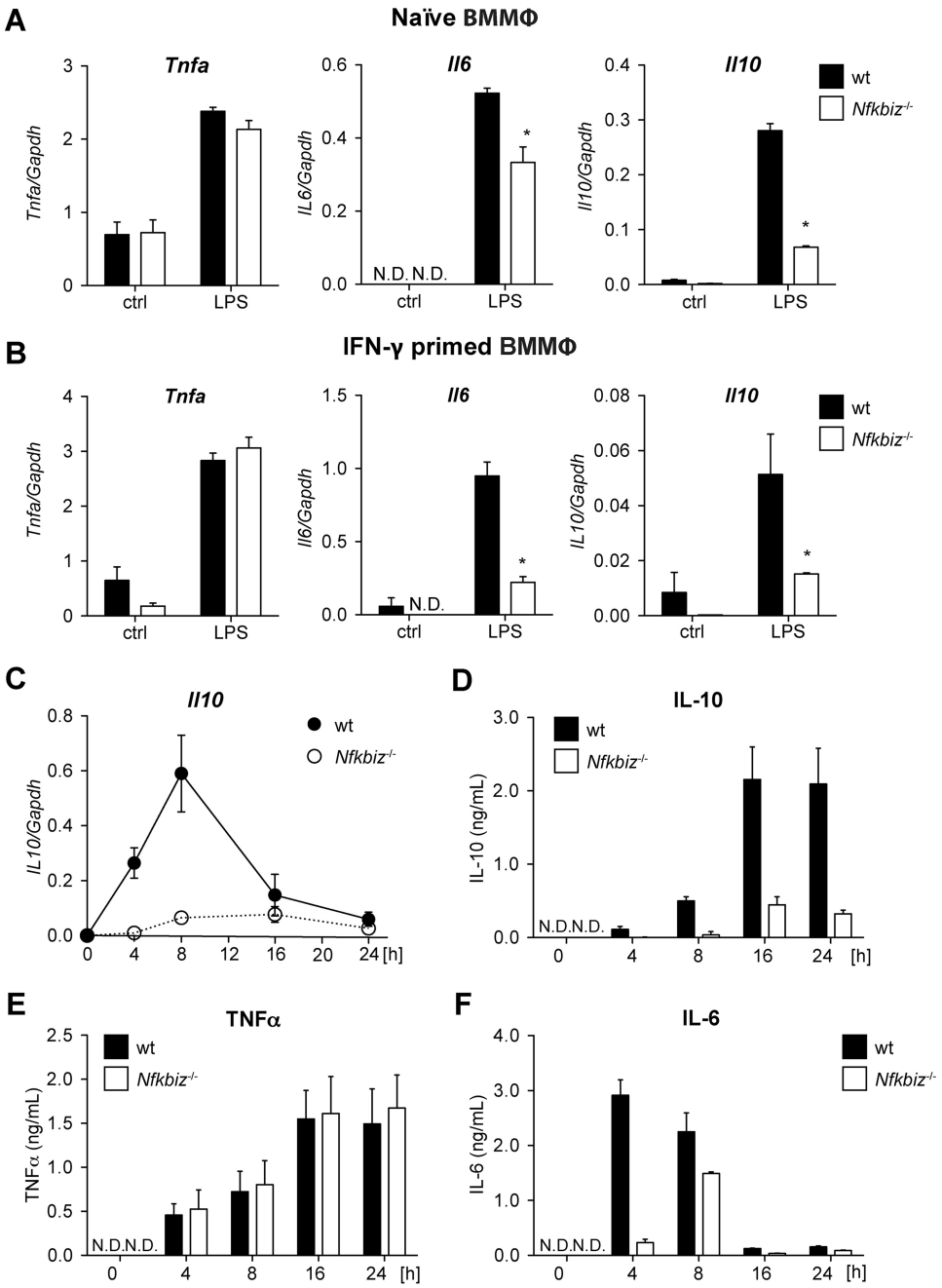

FIGURE 2. **IκBζ deficiency reduces IL-10 secretion in bone marrow macrophages.** *A* and *B*, naïve (*A*) and classically activated (*B*) wild-type and *Nfkbiz*$^{-/-}$ BMMΦ were cultured in the presence of 1 μg/ml LPS. Total RNA was subjected to qRT-PCR analysis to determine the expression levels of *Tnfa*, *Il6*, and *Il10*. Values are mean ± S.E. from five experiments. *ctrl*, control. *C*, wild-type and *Nfkbiz*$^{-/-}$ BMMΦ were cultured with 1 μg/ml LPS. Total RNA was isolated after the indicated time points and subjected to qRT-PCR analysis to determine the levels of *Tnfa*, *Il6*, and *Il10* mRNA expression. Values are mean ± S.E. from four experiments. *D–F*, IL-10 (*D*), TNFα (*E*), and IL-6 (*F*) concentrations in cell culture supernatants were assessed at the indicated time points of LPS stimulation. Values are mean ± S.E. from three experiments. *, statistical significance comparing wild-type and *Nfkbiz*$^{-/-}$ cells; *N.D.*, not detectable.

tion of *Il10* mRNA expression and protein secretion in macrophages.

IκBζ-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. 3, *A–C*). Interestingly, naïve *Nfkbiz*$^{-/-}$ macrophages secreted slightly more TNFα than wild-type BMMΦ (Fig. 3*A*). 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,

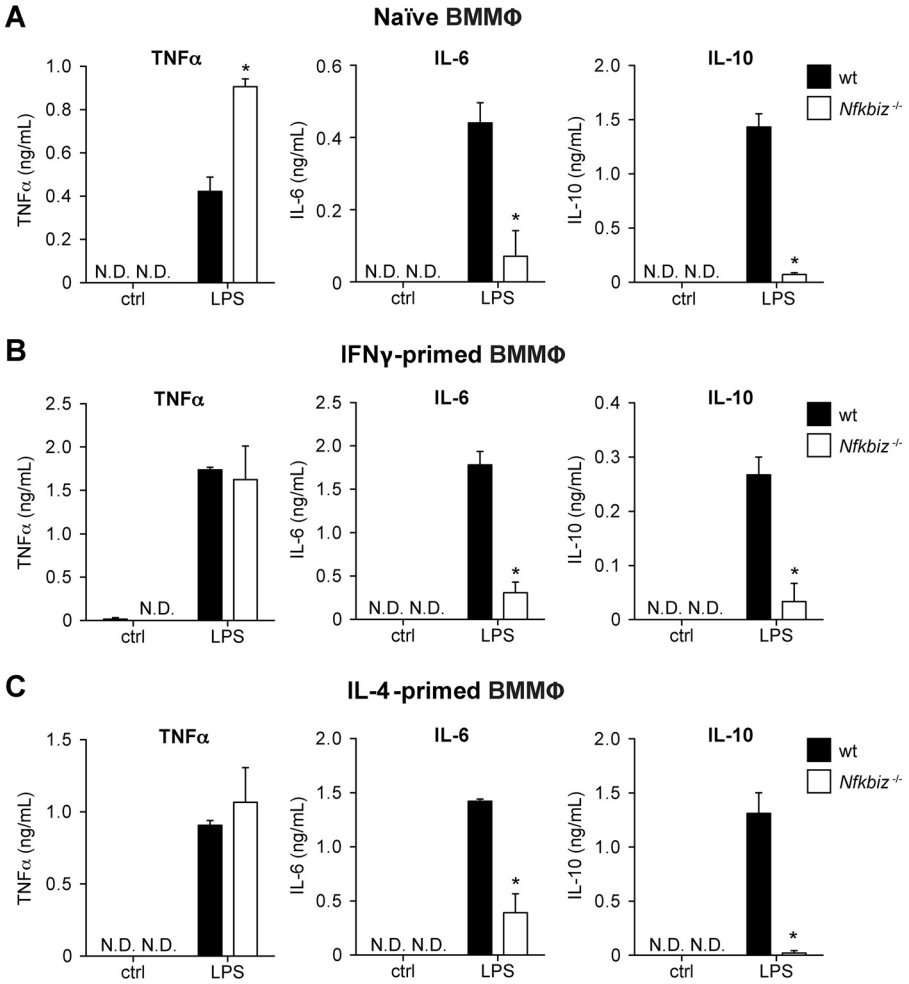

FIGURE 3. **IκBζ-regulated IL-10 expression is independent of macrophage polarization.** *A–C*, BMMΦ were either left naïve (*A*), classically activated with 30 ng/ml IFNγ (*B*), or alternatively activated with 20 ng/ml IL-4 (*C*) for 24 h before challenging or not with 1 μg/ml LPS for an additional 24 h. Culture supernatants were analyzed for the concentration of TNFα, IL-6 and IL-10. Values are mean ± S.E. of six experiments. *, statistical significance comparing luciferase activity in the presence and absence of doxycycline; *N.D.*, not detectable. *ctrl*, control.

expression of IL-10 is not affected by the macrophage polarization state.

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

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

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

IκBζ 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. 5*A*). Because these regions potentially serve as anchor points for IκBζ-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. 5*B*). Truncations of the *Il10* promoter and deletion of the distal

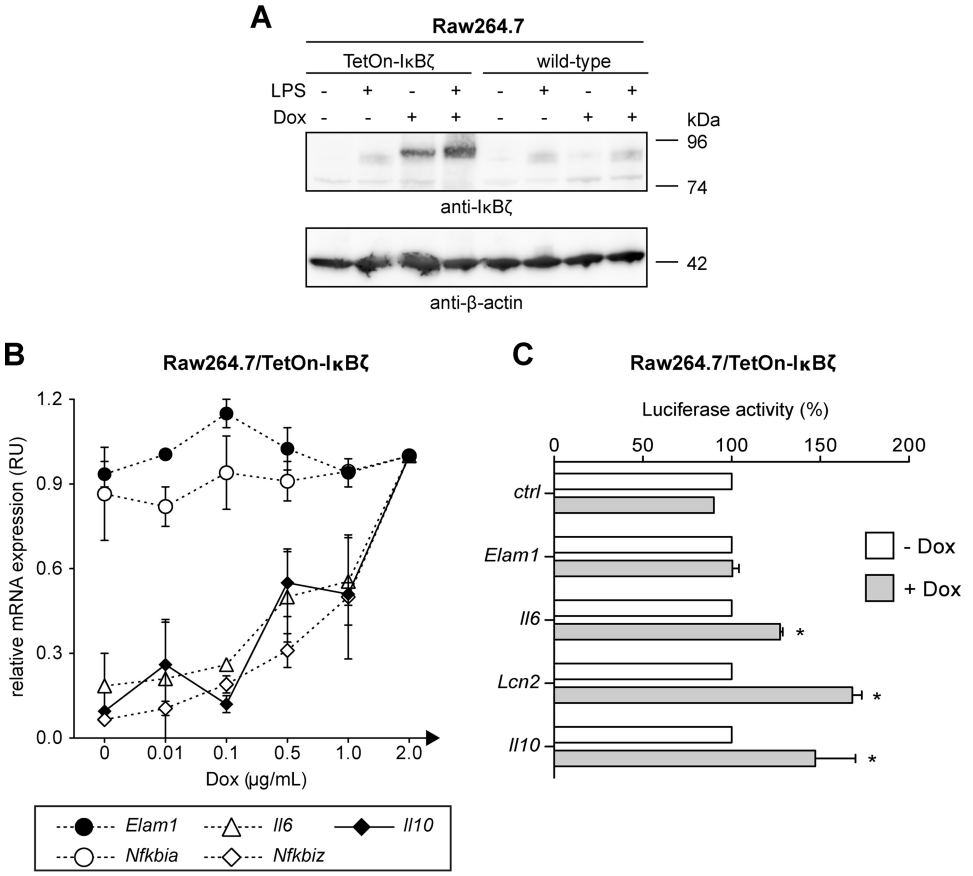

FIGURE 4. **Ectopic expression of IκBζ induces *Il10* expression.** *A*, Raw264.7 cells were modified to enable the inducible doxycycline-dependent expression of IκBζ. Both Raw264.7 wild-type and the resulting Raw264.7/TetOn-IκBζ cells were cultured in the presence or absence of either 1 μg/ml LPS, 2 μg/ml doxycycline (*Dox*), or a combination thereof. After 24 h, induction of IκBζ expression was verified by immunoblot analysis. *B*, Raw264.7/TetOn-IκBζ cells were incubated in the presence of the indicated amounts of doxycycline. After 24 h, total RNA was isolated and subjected to qRT-PCR analysis for the expression of *Il10*, *Il6*, *Nfkbiz*, *Nfkbia*, and *Elam1*. Values are expressed as relative units (*RU*). Expression levels from cells treated with 2 μg/ml doxycycline were defined as 1 RU. Values are mean ± S.D. from three experiments. *C*, Raw264.7/TetOn-IκBζ cells were transfected with luciferase reporter gene constructs harboring the promoter region of the indicated genes or the empty vector control (*ctrl*). Cells were cultured in the presence of 2 μg/ml doxycycline for 48 h before luciferase assay was performed. Luciferase activity is given as percentage of the basal activity in the absence of doxycycline. Values are mean ± S.D. from three experiments. *, statistical significance comparing luciferase activity in the presence and absence of doxycycline.

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 *Nfkbiz* did not result in luciferase activity (Fig. 5*C*). 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. 5*C*), 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 ($H3K4me_3$) 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 *Nfkbiz*. To this end, we employed qPCR-coupled ChIP analysis of the endogenous proximal *Il10*, *Il6*, and *Tnfa* promoter regions using an $H3K4me_3$-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. 5*D*). 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. 5*D*). 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 Nfkbiz$^{-/-}$ *BMM*Φ—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

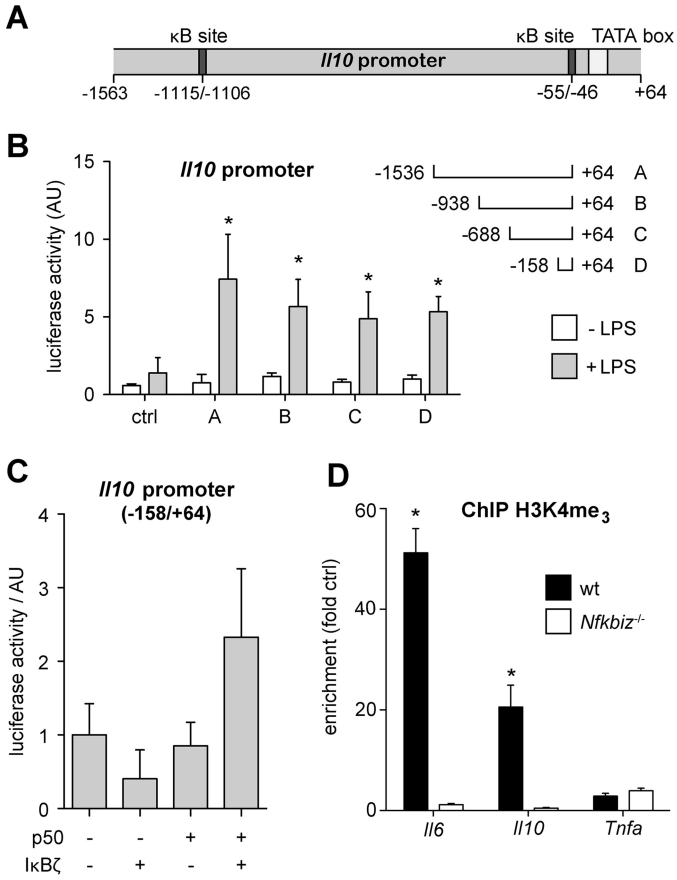

FIGURE 5. **IκBζ regulates *Il10* promoter activity.** *A*, schematic of the murine *Il10* promoter. The *dark boxes* indicate the positions of κB binding sites. *B*, Raw264.7 cells were transfected with the empty luciferase reporter gene vector (*ctrl*) or reporter gene constructs harboring the indicated 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) naïve wild-type and *Nfkbiz*$^{-/-}$ BMMΦ was subjected to ChIP assays applying an H3K4me$_3$-specific antibody. The degree of H3K4 trimethylation of the *Il10*, *Il6*, and *Tnfa* promoter 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 *Nfkbiz*$^{-/-}$ cells.

the pro-inflammatory phenotype of *Nfkbiz*$^{-/-}$ 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 *Nfkbiz*$^{-/-}$ 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 *Nfkbiz*$^{-/-}$ 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. 6*C*), whereas *Cxcl9* expression remained largely unaffected (data not shown). Importantly, when *Nfkbiz*$^{-/-}$ BMMΦ were incubated with IL-10-proficient supernatants from LPS-treated wild-type BMMΦ, a significant reduction of *Stat1* and *Gbp4* expression was also detected (Fig. 6*D*). 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. 3*A*). Thus, these results not only show that IL-10 signaling is functional in *Nfkbiz*$^{-/-}$ BMMΦ but, moreover, indicate that the lack of IL-10 in *Nfkbiz*$^{-/-}$ 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, *Nfkbiz*$^{-/-}$ 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 *Nfkbiz*$^{-/-}$ mice. These results were supported by the finding that the doxycycline-inducible expression of ectopic IκBζ in Raw264.7 macrophages tightly correlated with increasing *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

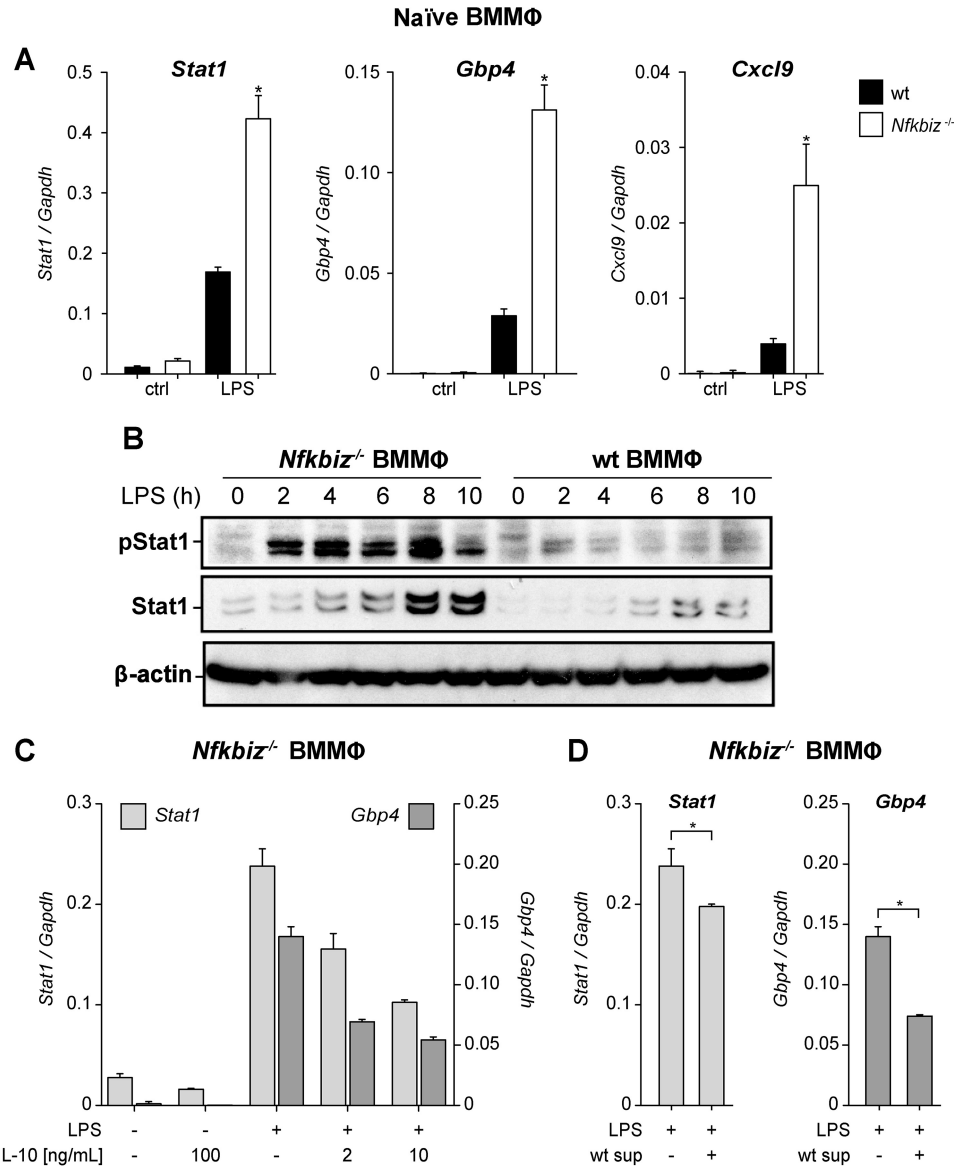

FIGURE 6. **IL-10 partially attenuates the M1-hyperpolarized state of *Nfkbiz*-deficient bone marrow macrophages.** *A*, BMMΦ from wild-type and *Nfkbiz*$^{-/-}$ mice were cultured in the presence or absence of 1 μg/ml LPS for 5 h. Cells were subsequently assayed for the indicated mRNA levels. Values were normalized to *Gapdh* and are mean ± S.D. from four experiments. *, statistical significance comparing wild-type and knockout cells; *N.D.*, not detectable. *ctrl*, control. *B*, wild type and *Nfkbiz*$^{-/-}$ BMMΦ were stimulated with 1 μg/ml LPS for the indicated times. The levels of Stat1, phospho-Stat1 (Tyr^{701}), and β-actin were analyzed by immunoblotting. An exemplary set of data is shown. *C* and *D*, *Nfkbiz*$^{-/-}$ BMMΦ were cultured in the presence of 1 μg/ml LPS or left untreated. After 4 h, the supernatants were replaced by medium supplemented with the indicated concentrations of IL-10 (*C*) or by supernatants from wild-type BMMΦ (*wt*) challenged for 24 h with LPS (*D*). Samples for qRT-PCR were prepared after 4 h of incubation and analyzed for relative mRNA expression of *Stat1* and *Gbp4*. Values are mean ± S.D. from three experiments.

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 $I\kappa B_{NS}$. For instance, although IκBζ is required for IL-6, IL-12p40, and G-CSF expression (15, 16), $I\kappa B_{NS}$ 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 *Il10* 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 *Il10* promoter (42). A recent study found that IκBζ recruits the epigenetic modifier Tet2 to selective promoter regions independent of DNA meth-

ylation (43). IκBζ 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 *Nfkbiz*$^{-/-}$ cells. Thus, it will be interesting to explore whether Bcl-3 and IκBζ 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 IκBζ is also relevant to other cell types. Our exemplary investigation of wild-type and *Nfkbiz*$^{-/-}$ MEFs indicates that the described mechanism is not restricted to macrophages. Interestingly, previous gene expression analysis revealed that IκBζ-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 *Nfkbiz*$^{-/-}$ 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 IκBζ-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 *Nfkbiz*$^{-/-}$ 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 secretion 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 *Nfkbiz*$^{-/-}$ 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 *Nfkbiz*$^{-/-}$ mice exhibit an increased susceptibility to dextran sodium sulfate-induced colitis.[4]

In summary, we have uncovered an essential novel regulatory mechanism of *Il10* gene regulation in macrophages. We demonstrate that IκBζ 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, *Nfkbiz*$^{-/-}$ mice are an interesting model system for evaluating IκBζ as a potential therapeutic target in inflammatory diseases.

[4] S. Hörber, D. G. Hildebrand, S. Lorscheid, J. S. Frick, K. Schulze-Osthoff, and F. Essmann, unpublished results.

Author Contributions—Se. H., D. G. H., K. S. O., and F. E. designed the research. Se. H., D. G. H., W. S. L., and S. L. performed the research. Se. H., D. G. H., W. S. L., and F. E. analyzed the data. St. H., K. S. O., and F. E. wrote the paper.

Acknowledgments—We thank S. Gaffen, M. Morimatsu, S. Smale, I. Schmitz, and G. Totzke for reagents.

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