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IkBζ Is a Transcriptional Key Regulator of CCL2/MCP-1

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CCL2, also referred to as MCP-1, is critically involved in directing the migration of blood monocytes to sites of inflammation. Consequently, excessive CCL2 secretion has been linked to many inflammatory diseases, whereas a lack of expression severely impairs immune responsiveness. We demonstrate that IkBζ, an atypical IkB family member and transcriptional coactivator required for the selective expression of a subset of NF-κB target genes, is a key activator of the Ccl2 gene. IkBζ-deficient macrophages exhibited impaired secretion of CCL2 when challenged with diverse inflammatory stimuli, such as LPS or peptidoglycan. These findings were reflected at the level of Cll2 gene expression, which was tightly coupled to the presence of IkBζ. Moreover, mechanistic insights acquired by chromatin immunoprecipitation demonstrate that IkBζ is directly recruited to the proximal promotor region of the Ccl2 gene and is required for transcription-enhancing histone H3 at lysine-4 trimethylation. Finally, IkBζ-deficient mice showed significantly impaired CCL2 secretion and monocyte infiltration in an experimental model of peritonitis. Together, these findings suggest a distinguished role of IkBζ in mediating the targeted recruitment of monocytes in response to local inflammatory events. The Journal of Immunology, 2013, 190: 4812–4820.

Resident tissue macrophages (Mφ) make up the first defense line against infiltrating pathogens. In these cells, pathogen recognition triggers a rapid and extensive cascade of physiological responses, including, besides others, the direct intruder abatement and release of proinflammatory cytokines that initiate a systemic immune response (1–3).

Among the most prominent cytokines, CCL2 (MCP-1), the first discovered member of the CC family of chemokines, mediates the chemotactic recruitment of circulating monocytes to inflammatory sites (4–7). Even though further signal molecules, such as CCL7 and CCL13, exhibit overlapping functions, CCL2 seems to be the only nonredundant monocyte-specific chemokine (3, 5, 8). Its expression in mice causes reduced susceptibility for inflammatory disorders, such as rheumatoid arthritis, multiple sclerosis, atherosclerosis, glomerulonephritis, or inflammatory bowel disease, where increased CCL2 levels have been linked to the onset or progression of disease (3, 8, 9). Strikingly, disruption of the Ccl2 gene in mice causes reduced susceptibility for inflammatory diseases (4, 5, 10, 11). As a consequence, almost all major pharmaceutical companies aim at developing antagonistic small molecules targeting the most relevant CCL2 receptor CCR2 for the therapy of chronic inflammation (12, 13).

Under noninflammatory conditions, the Ccl2 locus is transcribed at low level, but rapid induction of gene expression occurs on exposure of cells to various proinflammatory stimuli (14, 15). The differential Ccl2 gene expression is controlled at the promoter level by a complex network of transcriptional regulators, including NF-κB, C/EBP, AP-1, and Sp-1 (16). Despite such complexity, inflammation-linked, high-level expression appears to be dominantly regulated by NF-κB proteins (16–19). Recent research has shed new light on the mechanisms underlying transcriptional activation by NF-κB, which can regulate hundreds of different target genes. Current efforts focus on understanding of how only certain subsets of NF-κB target genes are selectively induced depending on the cell type, a given stimulus, and environmental parameters (20–22). Such insights may open up novel therapeutic strategies aiming to specifically modulate NF-κB responses in chronic inflammation (20).

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 inflammatory genes can be categorized in two groups, based on their kinetics of induction and the requirement of protein synthesis. Whereas primary response genes are rapidly induced, the expression of secondary target genes is delayed and requires the prior synthesis of additional NF-κB coregulators. In this context, IkBζ, an atypical IkB protein, has been recently identified and implicated in differential NF-κB target gene expression in Mφ (23–25), even though its physiological function remains largely unknown. The IkBζ-coding Nfkbia gene is rapidly induced as a primary NF-κB response gene by various inflamma-
tory stimuli. Upon de novo synthesis, protein interactions with p50 NF-κB homodimers seem to mediate the recruitment of IkBζ to target promoters (23, 26). IkBζ is believed to act as a transcriptional coactivator required for the expression of a subset of NF-κB secondary response genes, presumably by facilitating transcription-enhancing nucleosome remodeling (21, 27).

In this article, we identify the murine Cc12 gene as a novel target of IkBζ-regulated gene expression and show that IkBζ is critical for CCL2 secretion in Mφ. Furthermore, in vivo evidence in a model of experimental peritonitis suggests that this regulatory mechanism is relevant in systemic inflammation and necessary for the proper recruitment of monocytes to sites of inflammation.

Materials and Methods

Animals

*Nfkbiz* ko/ko (28) and littermate control mice were used at 6–18 wk of age. Mouse work was conducted 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 Mφ

Female C57BL/6 mice were euthanized by CO2 asphyxiation. For isolation of peritoneal Mφ (PMφ), the abdominal skin was removed, a catheter (24 gauge) was inserted into the peritoneal cavity, and 10 ml ice-cold PBS was injected. After massage of the peritoneum, peritoneal fluids were aspirated, centrifuged at 500 × g for 5 min, and yielded PMφ were resuspended in 0.5–1.5 ml Mφ medium containing DMEM/Hams F12, 10% FCS, and MycoZapPlusCL antibiotics (Lonza). Next, cells were seeded in 96-well plates (100,000 cells/well) and cultivated at 37˚C for 2 h. Finally, adherent cells were washed four times with culture medium to acquire purified PMφ. For isolation of bone marrow–derived Mφ (BMMφ), femur and tibia were separated and flushed with PBS, and cells were pelleted by centrifugation. After resuspension, 3 × 10⁶ cells/ml were seeded in uncoated tissue culture flasks and cultured under low oxygen conditions (5% CO₂, 5% O₂) in Mφ medium supplemented with murine M-CSF (30 ng/ml; Immunotools). After 7 d of differentiation, cells were washed twice with PBS. Adherent cells were scraped off and cultured in 96-well plates (2 × 10⁵ cells/cm²) under low oxygen conditions.

Activation and proinflammatory stimulation of PMφ and BMMφ

Isolated PMφ and differentiated BMMφ were incubated in the presence of 20 ng/ml murine IL-4 (Immunotools) or 30 ng/ml murine IFN-γ (Immunotools) for 24 h to achieve alternative or classical Mφ activation. For proinflammatory stimulation, media of activated and nonactivated cells were supplemented with 25 ng/ml murine IL-1β or TNF-α (Immunotools), 1 μg/ml LPS (Escherichia coli serotype O111:B4; Sigma), 1 μg/ml peptidoglycan (PGN; Bacillus subtilis; Sigma), or 1 μg/ml zymosan A (Zym; Sigma).

Generation of Raw264.7/TetOn-IκBζ cells

Raw264.7 cells were genetically modified to allow inducible expression of the canonical IκBζ isoform (IkBζL1; GenBank accession no. NM_001159394.1) using the pINDUCER system (29). In brief, IkBζL1 was amplified from murine cDNA applying the primers 5'-CACCAGGATCTGCTG-3' and 5'-CTAGTATGGTGCTCGG CTG-3'. The amplicon was cloned into pENTR-D-TOPO using TOPO cloning (Invitrogen), and the coding sequence was transferred to pINDUCER20 using gateway technology (Invitrogen). Upon sequence verification, lentivirus was generated using the Lenti-X system (Clontech). After lentiviral transduction, Raw264.7 cells were cultured in the presence of 500 μg/ml G418 for selection of Raw264.7/TetOn-IκBζ cells.

Culture and stimulation of Raw264.7 cells and mouse embryonic fibroblasts

Mouse embryonic fibroblasts (MEFs) were isolated at embryonic day 10.5 (E10.5) according to standard procedures (30). MEFs and Raw264.7 cells were cultured under low oxygen and standard conditions, respectively. The

![FIGURE 1.](http://www.jimmunol.org/)

**FIGURE 1.** LPS-triggered Cc12 expression is impaired in IkBζ-deficient PMφ. (A) Nonactivated PMφ from *Nfkbiz* ko/ko and *Nfkbiz* wt/wt mice were stimulated with 1 μg/ml LPS for 4 h or left untreated. Total RNA was isolated and subjected to microarray analysis to identify novel putative targets of IkBζ-dependent gene regulation. The heat map illustrates an exemplary subset of acquired data with representative members of the following three gene clusters: 1) putative NF-κB target genes, 2) housekeeping genes, and 3) NF-κB-regulated gene expression. Values are mean ± SEM from four to six experiments. (**p < 0.05, ***p < 0.005.)
latter additionally received 0.5 μg/ml G418 to maintain the transduced cell population. Cells were seeded at a density of 10^5 cells/cm² 24 h before the experiments. Optionally, 2 μg/ml doxycycline (Sigma) was added to induce ectopic IκBζ expression. Subsequently, cells were cultured in the presence of 1 μg/ml LPS for proinflammatory stimulation.

**Quantitative RT-PCR**

Whole-cell RNA was isolated using the RNeasy mini kit (Qiagen) and reverse-transcribed (Quantitect kit; Qiagen) as per manufacturer’s instructions. Quantitative PCR (qPCR) was performed in a LightCycler 480 II (Roche) using the Maxima Hot Start Taq DNA polymerase (Thermo) in the manufacturer’s two-step cycling protocol (384-well plates; 10 μl reaction). Transcripts were analyzed applying the following primer pairs: Gapdh (5’-ACCACAGTCCATGACTAC-3’, 5’-CACCACCTGTGCTGAG-CC-3’), Il6 (5’-AGTGGGCTTCTGGAGCTGA-3’, 5’-TCCAGATTTCCCAAGAACA-3’), Ccl2 (5’-GGAGGAAGCAGCCAGCAC-3’, 5’-TG- GGCGTGACTGACCTTCGG-3’), Nfkbic (5’-TATCGGTGACAGACATTTGGA-3’, 5’-TGATGAGGCTTCCTCCCTCAG-3’), Elaml (5’-CTCACTCTGACATGCTCC-3’, 5’-ACGTGGTAAGAAGGACATGG-3’), Tnfa (5’-CTCTGACTTTCCCTTCCTCT-3’, 5’-GGTTGAGGTGAAGGACAGA-3’). Quantification of reverse-transcribed mRNA was performed using the second derivative maximum-based “Advanced relative quantification algorithm” of Roche’s LightCycler 480 Software (V1.5).

**Affymetrix analysis**

Total RNA was isolated from nonactivated PMφ, which were either left untreated or stimulated with 1 μg/ml LPS for 4 h, and processed for hybridization on Affymetrix MoGene 1.0 ST arrays according to the manufacturer’s instructions. Single-strand cDNA was prepared using the Ambion WT Expression kit and labeled using the GeneChip WT Terminal Labeling kit. Hyridization, scanning, and generation of probe cell intensities were performed on an Affymetrix GeneChip fluidics station 450 and GeneChip Scanner 3007 G using Affymetrix Expression Console 1.1 software. Identification of LPS-regulated genes and comparison of LPS-induced gene expression in wild-type (wt) and knockout cells were performed as described previously (31). Data are published in the GEO database (GEO accession no. GSE43075; http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=rvhhbhumikjkwj&acc=GSE43075).

**Immunoblotting**

Immunoblotting was performed as described previously (32). Anti-β-actin mAb was purchased from Sigma (clone AC-74; 1:2000). Polyclonal rabbit anti-IκBζ Ab was raised against a combination of peptides corresponding to short sequences of murine IκBζ (N-PSGSPGSSDFSTSSVSC-C, N-QRRILGKSIQKRAPPY-C), affinity-purified with these peptides, and used at a concentration of 0.5 μg/ml.

**Chromatin immunoprecipitation**

A total of 10^7 cells/sample were stimulated with 1 μg/ml LPS for indicated periods. Chromatin immunoprecipitation (ChIP) was performed with Diagenode’s Red ChIP kit using polyclonal rabbit anti-IκBζ or anti-histone H3 (trimethyl K4; Abcam). Abs. ChIP efficiencies were determined by qPCR on a LightCycler 480 II (Roche) using the Maxima Hot Start Taq DNA Polymerase in the manufacturer’s two-step cycling protocol (96-well plates; 20 μl volume). The following primer pairs spanning indicated promoter regions of the murine Ccl2 gene were used: Ccl2_1306.3 (5’-TCA(A/C)CA(A/T)TTC-3’, 5’-CTCTGACATTTCCCTTCCT-3’), Ccl2_2501.2303 (5’-CCTTGTTGTGACTTCCCAGATGT-3’, 5’-TCTGTCACAACAAAGGAGGATT-3’). Primers covering portions of Aktb (5’-TCTGATACACACGAGACATCC-3’, 5’-GCCAGATTTTTAATCCTCGCC-3’) and Gapdh (5’-TACCTAGGGTTTTACGGGCG-5’) were used as controls. Specificities of the primers were verified and PCR efficiencies were determined. Analysis was performed according to manufacturer’s standard procedures. In brief, treatment-specific ChIP efficiencies, expressed as percentage of input, were first determined via the formula: Eff = d^-1((1+E)^d)  

**qPCR analysis**

Quantification of reverse-transcribed mRNA was performed using the second derivative maximum-based “Advanced relative quantification algorithm” of Roche’s LightCycler 480 Software (V1.5).

**FIGURE 2**. The impact of IκBζ on Ccl2 gene expression is conserved among murine cell lines and cell types. (A) Raw264.7 cells (Ctrl) were modified to enable the inducible doxycycline-dependent expression of the canonical isoform of IκBζ [IκBζ(L)], yielding the cell line Raw264.7/TetOn-IκBζ (assigned IκBζ). Both cell lines were cultured in presence or absence of 2 μg/ml doxycycline for 24 h to verify the induction of IκBζ by immunoblot analysis using Abs specific for IκBζ and β-actin (upper panel). Moreover, Raw264.7/TetOn-IκBζ Mφ were incubated in the presence of indicated increasing amounts of doxycycline for 24 h to induce IκBζ in a dose-dependent manner (lower panel). Afterward, total RNA was isolated and subjected to qRT-PCR analysis of Ccl2, Il6, Nfkbic, Nfkbia and Elaml levels (expressed as relative units [RU]; expression levels from cells treated with 2 μg/ml doxycycline were defined as 1 RU). In addition, supernatants were analyzed by triplicate for secreted amounts of CCL2 (secretion of untreated cells was defined as 1 RU). Representative data sets of qRT-PCR and immunoblot analyses or means ± SD of the cytokine measurements are shown for three experiments. (B and C) BMMΦ (nonactivated, B) or MEFs (C) from Nfkbic^+/+ and Nfkbic^ko/ko animals were cultured in the presence of 1 μg/ml LPS for 4 h. Total RNA was subjected to qRT-PCR analysis to determine levels of Nfkbic, Tnfa, Ccl2, and Il6 expression. Values are means ± SEM for four (BMMΦ) or eight (MEFs) experiments, respectively. Asterisks indicate statistical significance comparing Nfkbic^+/+ and Nfkbic^ko/ko cells. *p < 0.05, **p < 0.005.
cies, which define the specific ratio of a distinct signal over the background and are expressed as “Fold Ctrl,” were determined by the equation: O = EffLocus,Treatment/(EffGapdh,Treatment + EffActb,Treatment)^2, where O is treatment- and locus-specific relative occupancy and EffLocus,Treatment is locus- and treatment-specific ChIP efficiency.

Quantification of cytokines
Concentrations of CCL2, TNF-α, and IL-6 in peritoneal fluids and culture supernatants were determined by Cytometric Bead Arrays (Becton-Dickinson). Before stimulation of cells in 96-well plates, the culture medium was exchanged with 200 μl fresh medium per well. In case of BMMφ, cytokine concentrations in culture supernatants were directly compared and expressed as cytokine amounts per volume. Because of donor 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).

Experimental peritonitis
Male Nfkbia−/− and control mice were used at 6–18 wk of age. Animals received an i.p. injection of 500 μl PBS containing 40 μg Zym (Sigma) per gram body weight. Mice were sacrificed 4 h later by CO2 asphyxiation. Subsequently, peritoneal cavity fluids were harvested and analyzed for cytokine amounts. In addition, peritoneal cells were FACS-phenotyped as described previously (32). After centrifugation (500 g, 5 min), peritoneal cells were resuspended in 10 ml FACS buffer (PBS containing 2% FCS), pelleted and resuspended in FACS Lysing solution (Becton Dickinson) to block Mφ FcRs CD16 and CD32. After 10 min of incubation on ice, an equivalent volume of FACS buffer containing the following fluorochrome-labeled Abs at the indicated dilutions was added: rat anti-mouse F4/80:PE (A3-1; 1:20; AbD), rat anti-mouse CD19:FITC (AbD; 6D5; 1:20), rat anti-mouse Gr-1: Pacific Blue (RB6-8C5; 1:50; BioLegend), rat anti-mouse CD11b:allophycocyanin (M1/70; 1:50; Becton Dickinson), hamster anti-mouse CD11c: allophycocyanin-Cy7 (HL3; 1:50; Becton Dickinson), and rat anti-mouse CD117:PE-Cy5 (2B8; 1:50; Becton Dickinson). After 20 min, cells were pelleted and resuspended in FACS Lysing solution (Becton Dickinson) to lyse RBCs, washed twice in FACS buffer, counted, and analyzed on an LSRII flow cytometer (Becton Dickinson). BD CompBead (Becton Dickinson) standard operating procedures were used to calculate color compensation parameters. Data were acquired using the WEASEL software (The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia) and evaluated applying the gating scheme outlined in Supplemental Fig. 1 to determine the relative proportion of respective cell types.

Statistical analysis
Values are given as mean ± SD or mean ± SEM. For statistical comparisons, hypotheses were tested by applying unpaired standard t tests. Asterisks indicate statistical significance (*p < 0.05, **p < 0.005, ***p < 0.0005).

Results
Identification of Ccl2 as a potential target gene of IkBζ
Even though IkBζ (Nfkbia) was shown to be expressed in the monocyte cell lineage upon proinflammatory stimulation, its physiological relevance remains largely unclear. To elucidate novel aspects of IkBζ function, we focused on its impact on Mφ physiology. Initially, PMΦ and BMMΦ from wt and Nfkbia−/− mice were assayed for basic cell-type features, such as phagocytosis, oxidative burst, or chemotactic responsiveness, which did not differ between the genotypes (data not shown). Upon activation with LPS, however, the proinflammatory mediator CCL2, a well-characterized IkBζ target gene (31), was induced in a statistically significant manner (p < 0.005) in the absence of IkBζ (Fig. 3). This suggests that IkBζ negatively regulates Ccl2 expression in Mφs, which is consistent with the notion that IkBζ functions to inhibit NF-κB transcriptional activity and thereby favors inflammatory gene repression, even though IkBζ−/− mice are generally more susceptible to bacterial, allergic, and autoimmune challenges in comparison to wt mice (31). Because Ccl2 is a well-characterized proinflammatory mediator with physiological and pathological relevance (33), we focused on its expression in Mφs in the following experiments. Thus, we subjected PMφs from wt and Nfkbia−/− mice to qPCR-ChIP analyses applying H3K4m3-specific Ab and primers spanning indicated genomic regions of the Ccl2 locus or portions of Actb and Gapdh genes, coding for β-actin and GAPDH, which served as control genes not targeted by IkBζ. Values are mean ± SD from three experiments. ChIP analyses with isotype control Abs were performed (data not shown) to exclude experimental artifacts. (A) Chromatin from LPS-treated Raw264.7 cells (2 h) and untreated control cells was analyzed by qPCR-ChIP assays using IkBζ-specific Ab and primers spanning indicated genomic regions of the Ccl2 locus or portions of Actb and Gapdh genes, coding for β-actin and GAPDH, which served as control genes not targeted by IkBζ. Values are mean ± SD from three experiments. ChIP analyses with isotype control Abs were performed (data not shown) to exclude experimental artifacts. (C) Chromatin from LPS-treated Nfkbia−/− and Nfkbia−/− nonactivated BMMΦ (5 h), as well as untreated control cells (Ctrl), was subjected to qPCR-ChIP assays applying H3K4m3-specific Ab. Primer Ccl2 (I) was used to determine the degree of H3K4 trimethylation of the Ccl2 promoter. Values are mean ± SD from three experiments. ChIP analyses with isotype control Abs were performed (data not shown) to exclude experimental artifacts. Values are given as mean ± SD or mean ± SEM.
LPS-inducible genes were expressed to a lower extent in nonregulated by IκBζ. At the same time, inducibility of NF-κB function of IκBζ as a transcriptional coactivator (23), numerous LPS-inducible genes were expressed to a lower extent in Nfkbiz−/− PMΦ, including Lcn2, Edn2, Il6, Il12b, Csf2, and Csf3 (Fig. 1A). At the same time, inducibility of NF-kB target genes supposedly not regulated by IκBζ, including Ccl1 (27), Ccl2 (27), and Tnfa (23), did not significantly differ between both genotypes. Accordingly, the approach seemed to allow the identification of several new putative target genes of IκBζ (e.g., Ccl7, Cxcl5, and Ljp) showing lower expression in stimulated knockout PMΦ. Our special interest hereafter focused on the Ccl2 locus, because CCL2 secretion is essential for monocyte recruitment during the onset of inflammation (4) and is, therefore, directly linked to the guardian function of MΦ in peripheral tissues.

**IκBζ expression correlates with Ccl2 expression in LPS-stimulated cell lines and types**

Previous analyses on Nfkbiz-dependent gene expression had been performed in classically (IFN-γ) activated PMΦ (23). To confirm Ccl2 as an IκBζ target gene, we therefore determined the kinetics of induction of Ccl2 mRNA levels and CCL2 cytokine levels in IFN-γ-activated PMΦ in response to LPS by quantitative RT-PCR (qRT-PCR; Fig. 1B) and cytometric bead assay assays (Fig. 1C), respectively. TNF-α, which is supposedly regulated independently of IκBζ, was expressed at comparable levels in wt and Nfkbiz−/− PMΦ. In contrast, regardless of LPS stimulation, the mRNA levels of Ccl2 were significantly lower in IκBζ-deficient PMΦ and, moreover, exhibited altered induction kinetics with mRNA levels peaking earlier compared with wt cells. Similar characteristics were observed for Il6 that served as control for IκBζ-targeted gene regulation. Consistently, CCL2 levels in supernatants of Nfkbiz−/− PMΦ were ∼6-fold higher than in knockout supernatants, whereas amounts of TNF-α did not differ (Fig. 1C).

To determine whether this dependency of Ccl2 expression on the Nfkbiz−/−-genotype is conserved among MΦ, we included the MΦ-like Raw264.7 cell line and BMMΦ into our analysis. Raw264.7 cells were genetically modified to allow the doxycycline-inducible expression of the canonical isoform of IκBζ (IκBζdDox; Fig. 2A, upper panel). In Raw264.7/TetOn-IκBζ cells, doxycycline treatment induced Nfkbiz mRNA levels tightly correlating with increased mRNA levels of Ccl2 (maximum: ∼30-fold) and Il6, whereas the IκBζ-independent NF-κB target genes Elam1 and Nfkbia were not expressed to a higher degree (Fig. 2A, lower panel). This regulation of Ccl2 was also reflected at the level of CCL2 secretion, which was ∼5-fold induced upon doxycycline-triggered IκBζ expression (Fig. 2A, lower panel).

Similar to PMΦ, BMMΦ from wt and Nfkbiz−/− mice were stimulated with LPS and compared with respect to mRNA expression of Ccl2, Il6, and Tnfa (Fig. 2B). The inducibility of Ccl2 and Il6 was massively impaired in Nfkbiz−/− BMMΦ, whereas Tnfa expression did not significantly differ in both genotypes. Finally, MEFs isolated from Nfkbiz−/− mice revealed a similar

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**FIGURE 4. Ccl2 gene expression and CCL2 secretion are generally impaired in Nfkbiz−/− PMΦ in response to proinflammatory stimulation.** wt and Nfkbiz−/− PMΦ, classically activated with IFN-γ, were cultured with 1 μg/ml PGN (A). 1 μg/ml Zym (B), 25 ng/ml IL-1β (C), or 25 ng/ml TNF-α (D). Total RNA was isolated after the indicated time points and subjected to qRT-PCR analysis to determine levels of Nfkbiz, Tnfa, Ccl2, and Il6 mRNA expression. Values are mean ± SEM from five to six experiments. (E) Culture supernatants were assayed for levels of CCL2, IL-6, and TNF-α after 24 h. Cytokine levels are given as amount of cytokine per total protein. Values are mean ± SEM for six experiments. Asterisks indicate statistical significance in a t test comparing Nfkbiz+/- and Nfkbiz−/− cells: *p < 0.05, **p < 0.005, ***p < 0.0005. BD, Below limit of detection; ND, not determined.
pattern of gene regulation and showed significantly lower levels of Ccl2 mRNA after LPS stimulation as compared with Nfkbiz^{wt/wt} cells (Fig. 2C).

**IkBζ targets the proximal promoter region of the Ccl2 genomic locus**

Because our data clearly indicated an influence of IkBζ on Ccl2 transcription, we next investigated a direct interaction of IkBζ with the Ccl2 promoter. Two distinct promoter portions have been shown to be relevant in Ccl2 gene regulation: a proximal promoter region and a distal enhancer portion, both of which harbor NF-κB–binding sites that may serve as anchor points for the recruitment of IkBζ (Fig. 3A).

We first performed qPCR-coupled ChIP (qPCR-ChIP) analysis in LPS-stimulated and untreated Raw264.7 cells (Fig. 3B). The relative occupancy of the Ccl2 locus was not increased using an IkBζ-specific Ab compared with negative control loci (Gapdh, Actb) in unstimulated cells exhibiting no or minimal IkBζ expression. In contrast, the occupancy of the proximal promoter region was ~2.5-fold higher compared with the ones of control loci in the presence of LPS-induced IkBζ. Importantly, no enrichment was detected at the distal enhancer region in these samples, indicating that IkBζ was specifically recruited to the proximal Ccl2 promoter region.

The trimethylation of histone H3 at lysine-4 (H3K4m3), a histone signature of open chromatin, is linked to promoter regions with active transcription. We therefore investigated H3K4m3 occupancy of the proximal Ccl2 promoter region. qPCR-ChIP analysis revealed increased H3K4 trimethylation after LPS stimulation of BMMΦ from Nfkbiz^{wt/wt} mice, which was absent in non-stimulated cells (Fig. 3C). Interestingly, almost no binding of H3K4m3 was found after LPS treatment of Nfkbiz^{ko/ko} BMMΦ, indicating that the transcription-enhancing H3K4 trimethylation at the Ccl2 promoter depends on IkBζ.

**FIGURE 5.** Nfkbiz^{ko/ko} Mφ exhibit impaired CCL2 secretion independent of their state of activation. Differentially activated Nfkbiz^{wt/wt} and Nfkbiz^{ko/ko} PMΦ (A) or BMMΦ (B) were cultured in the presence of 1 μg/ml LPS, 25 ng/ml IL-1β, 25 ng/ml TNF-α, 1 μg/ml PGN, 1 μg/ml Zym, or left untreated (Ctrl) for 24 h. Starting 24 h before stimulation, cells received 30 ng/ml IFN-γ or 20 ng/ml IL-4 to induce classical and alternative activation, respectively. Naive Mφ were left untreated. Culture supernatants were assayed for levels of CCL2, IL-6, and TNF-α. Cytokine levels are given as amounts of cytokine per total protein (PMΦ) or per volume of supernatant (BMMΦ). Values are mean ± SEM from six experiments. Asterisks indicate statistical significance in a t test comparing Nfkbiz^{wt/wt} and Nfkbiz^{ko/ko} cells: *p < 0.05, **p < 0.005. BD, Below limit of detection; ND, not determined.
Role of the proinflammatory stimulus and Mφ polarization on IkBζ-induced cytokine expression

Expression of IkBζ is not restricted to LPS stimulation but can be induced in response to several activators of NF-κB, and especially of TLR/IL-1 signaling (23, 24). To investigate whether expression of Ccl2 and CCL2 secretion were generally impaired in IkBζ-deficient cells, we analyzed the kinetics of Nfkbi-z and Ccl2 induction in IFN-γ–activated PMΦ in response to several proinflammatory stimuli, including PGN, Zym, IL-1β, and TNF-α. PGN (Fig. 4A) and Zym (Fig. 4B) strongly induced Nfkbi-z, as well as IL6 and Ccl2. The mRNA induction of both cytokines, but not of Tnfa, was significantly reduced in Nfkbi-zko/ko cells when compared with wt PMΦ. Induction of Nfkbi-z and the cytokines was significantly lower in response to TNF-α (Fig. 4C) and IL-1β (Fig. 4D), but even under these conditions an impaired Ccl2 expression was observed in IkBζ-deficient cells. Consistent results were obtained at the level of cytokine secretion, when culture supernatants of cells were assayed after 24 h of stimulation (Fig. 4E).

The previous experiments used PMΦ that were classically activated with IFN-γ, which induces Mφ M1 polarization. Naive PMΦ and PMΦ activated with IL-4 to induce alternative (M2) polarization were assayed for cytokine secretion after 24 h of stimulation with LPS, PGN, IL-1β, and TNF-α, respectively, to investigate whether gene-regulatory effects of IkBζ are dependent on Mφ polarization (Fig. 5A). The same experiments were also performed with differentially activated BMMΦ (Fig. 5B). Moreover, stimulation of PMΦ with Zym was performed, as that trigger was later used for in vivo experiments. Interestingly, whereas IL-6 production strictly depended on IkBζ in classically (IFN-γ)-activated PMΦ (Fig. 4E), in naive and IL-4–primed PMΦ, IL-6 secretion occurred largely independently of IkBζ (Fig. 5A). Unlike PMΦ, BMMΦ revealed a strict IkBζ dependence of IL-6 secretion after stimulation with LPS and PGN, regardless of their polarization status (Fig. 5B). With respect to CCL2, cytokine secretion was strongly impaired in Nfkbi-zko/ko PMΦ, as well as in BMMΦ, regardless of whether experiments were conducted in naive, classically activated, or alternatively activated cells. Secretion of TNF-α, which is not directly regulated by IkBζ, was not impaired but generally even slightly increased in the absence of IkBζ. Altogether, these results demonstrate that CCL2 secretion, if triggered by a proinflammatory stimulus, is strictly dependent on IkBζ in both PMΦ and BMMΦ in each state of activation. In contrast, for other cytokines such as IL-6, the requirement of IkBζ is influenced by the Mφ type and their activation status.

In vivo evidence in a model of experimental peritonitis

CCL2 has originally been identified to be necessary for the inflammatory recruitment of monocytes in several models of experimental peritonitis (4–6). Thus, to investigate whether IkBζ is also required for the regulation of Ccl2/CCL2, we used an experimental peritonitis model in IkBζ-proficient and -deficient mice (Fig. 6A). This seemed reasonable, because the induction of endogenous CCL2 in this system is highly Mφ dependent (6). To induce systemic inflammation, we injected mice with Zym into the peritoneal cavity, and 4 h later, levels of TNF-α, IL-6, and CCL2, as well as quantities of monocyte infiltration, were determined. FACS phenotyping of peritoneal cells. Values are mean ± SEM from five experiments. Asterisks indicate statistical significance comparing Nfkbi-zwt/wt and Nfkbi-zko/ko mice: *p < 0.05. BD, Below detection limit.

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 each NF-κB target gene has an individual expression profile regarding kinetics, cell type, stimulus specificity, or requirement of cofactors, which is thought to ensure the selectivity of an immune response. A comparison of global gene expression patterns of LPS-stimulated Nfkbi-zwt/wt and Nfkbi-zko/ko PMΦ identified, among other genes, Ccl2 as a strongly inducible target of IkBζ-mediated transcription. To verify this finding, we challenged PMΦ and BMMΦ with various proinflammatory activators for the analysis of Ccl2 gene expression and CCL2 secretion. Our experiments consistently revealed that Nfkbi-zko/ko Mφ exhibited profoundly impaired Ccl2 expression and CCL2 secretion, in particular, when stimulated with TLR ligands. At the level of gene transcription, similar observations were made in untreated cells or after stimulation with IL-1β or TNF-α, which elicit only weak inflammatory responses. These findings were supported by the observation that the inducible expression of ectopic IkBζ in Raw264.7 cells tightly correlated with increasing Ccl2 mRNA levels. Thus, our results in knockout and overexpression models suggest that transcriptional regulation of Ccl2 directly depends on IkBζ.

IkBζ has been shown to exert its gene- activating effects largely on so-called secondary response genes. In contrast with primary NF-κB target genes that are immediately induced in the absence of new protein synthesis, secondary response genes require the prior synthesis and coactivator function of IkBζ, which itself is a primary NF-κB target gene (21, 23, 27). Consequently, these genes exhibit a delayed kinetics of gene induction. Consistent with this model, the kinetics of gene induction obtained in LPS-stimulated Raw264.7 cells and PMΦ could clearly discriminate between Nfkbi-z and Tnfa as IkBζ-independent primary response
genes on one side, and IL-6 and Ccl2 as IκBζ-dependent secondary response genes on the other hand. Maximal expression of IL-6 and Ccl2 was delayed but prolonged in Nfkbiw−/− cells. In contrast, in the absence of IκBζ, Ccl2 and IL-6 expression were considerably impaired and not sustained, but rather resembled Tnfα and Nfkbiw−/− in the kinetics of gene induction (Fig. 1B). In addition, our ChIP analyses revealed that IκBζ was directly recruited to the proximal region of the Ccl2 promoter, which harbors a single NF-κB binding site. The recruitment of IκBζ was associated with histone H3K4 trimethylation of the proximal promoter region as a marker of active transcription. Interestingly, in the absence of IκBζ, H3K4 trimethylation did not occur, which, together with other lines of evidence, suggests that chromatin remodeling events are essential for IκBζ action.

CCL2 expression is essentially required for monocyte recruitment to sites of inflammation and has therefore been implicated in (auto)inflammatory diseases, as well as in the course of immune defense against pathogens. This ambivalence of beneficial and pathogenic effects of CCL2 underscores the requirement of a tight regulation of proinflammatory factors for an appropriate immune response. To investigate the role of IκBζ-mediated CCL2 secretion in vivo, we chose a model of experimental peritonitis in which CCL2 secretion is predominantly driven by PMΦ (6). Our results demonstrate that knockout animals indeed exhibit a decreased capacity to secrete CCL2, as well as IL-6, and thus provide further evidence for the tight control of both cytokines by IκBζ. Interestingly, whereas IL-6 and CCL2 expression were strongly reduced in IκBζ-deficient mice, TNF-α levels were not decreased but even increased in comparison with wt animals. Similar findings were observed in PGN-stimulated BMMΦ and PMΦ from Nfkbiw−/− animals and might be caused by the fact that IκBζ can additionally function as a specific inhibitor of particular target genes (23).

Although cells of the monocyte/Mφ lineage are the major source of CCL2, several other cell types, including fibroblasts and epithelial and endothelial cells, can express CCL2 (16). Further investigations are necessary to verify whether the strict control of CCL2 by IκBζ is also relevant in these cell types. Our exemplary investigation of wt and Nfkbi−/− MEFs indicates that the described mechanism is not restricted to Mφ. Interestingly, however, we found that, unlike CCL2, the regulation of IL-6 by IκBζ might be also dependent on cell type-specific differentiation. For instance, whereas IL6 expression was strongly impaired in IκBζ-deficient BMMΦ, regardless of their polarization status, only M1 (IFN-γ)-polarized but not naive or M2 (IL-4)-polarized PMΦ revealed a profound inhibition of IL6 expression in the absence of IκBζ. Thus, this observation indicates that the assignment of a particular gene as an IκBζ-dependent secondary response gene might be also dependent on the cell type and specific chromatin context. Because our study did not aim to investigate the involvement of IκBζ in Mφ polarization, further questions concerning this issue have not been addressed in this article. However, our data may not only reflect the fact that Mφ polarization influences IκBζ-dependent gene regulation, but that vice versa IκBζ could have an impact on Mφ activation.

In future studies, it will be interesting to investigate the physiological impact of IκBζ-dependent Ccl2 regulation in different (patho)physiological contexts. Our results obtained in the experimental peritonitis model indicate that IκBζ deficiency causes impaired monocyte recruitment because of reduced CCL2 secretion. Nevertheless, this dependency may not exclusively be caused by defective CCL2 production. Given the fact that IκBζ influences the expression of several inflammatory key players, including IL-6, GM-CSF, G-CSF, IL-12p40, and IL-17, additional effects may influence homeostasis and trafficking of monocytes. IL-6, for example, has been shown to orchestrate the switch from neutrophil to monocyte recruitment during acute inflammation. In that context, the observation that TNF-α levels in peritoneal cavity fluids were dramatically higher in case of Nfkbiw−/− mice upon i.p. injection of Zym is interesting, because Nfkbiw−/− and Nfkbi−/− PMΦ did not exhibit such differences in vitro with respect to TNF-α secretion. Similar, but so far unexplained, effects have been observed previously (23) and might be secondary because of the downregulation of IκBζ-dependent genes regulating the expression of TNF-α in certain cell types. Differences in gene expression of additional cell type contributing to the outcome of the systemic peritonitis model might therefore explain this phenomenon. Thus, the ability of IκBζ to concertedly regulate various cytokines and physiological functions might distinguish IκBζ as a key transcriptional regulator of certain inflammatory processes (34). Moreover, other biological processes, such as Tq2 polarization (35), bone morphogenesis (16), or tumor progression (9, 16, 36) have been shown to be regulated by CCL2 and could be controlled by IκBζ.

In summary, we have uncovered an essential regulatory mechanism of Ccl2 gene regulation in Mφ. We demonstrate that IκBζ, presumably by the p50 NF-κB-mediated recruitment to the proximal Ccl2 promoter and subsequent histone modification, enables transcription of the Ccl2 locus, thus allowing production of high levels of CCL2 in the course of proinflammatory processes. As indicated by observations in an in vivo model of systemic inflammation, this IκBζ-dependent mechanism may be crucial for Mφ physiology, for example, in the course of Mφ-mediated effector cell recruitment.

Disclosures
The authors have no financial conflicts of interest.

References


