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IL-1β-Specific Up-Regulation of Neutrophil Gelatinase-Associated Lipocalin Is Controlled by IκB-ζ[1]

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Neutrophil gelatinase-associated lipocalin (NGAL) is a siderophore-binding protein that exerts a bacteriostatic effect by sequestering iron. Strong induction of NGAL synthesis has been observed in inflamed epithelium of the lungs and colon. Expression of NGAL is up-regulated in the lung epithelial cell line A549 by IL-1β, but not by TNF-α, despite an induction of NF-κB binding to the NGAL promoter by both cytokines. In this study, we present evidence that the IL-1β specificity is caused by a requirement of the NGAL promoter for the NF-κB-binding cofactor IκB-ζ for transcriptional activation. Up-regulation of NGAL expression in A549 cells following IL-1β stimulation was dependent on de novo protein synthesis and was greatly diminished by a small interfering against IκB-ζ mRNA. Cotransfection of A549 cells with a plasmid expressing IκB-ζ made TNF-α capable of inducing NGAL transcription, indicating that IκB-ζ induction is the only factor discriminating between IL-1β and TNF-α in their ability to induce NGAL expression. Coexpression of the cofactor Bcl-3, which is closely related to IκB-ζ, did not enable TNF-α to induce NGAL transcription. A functional NF-κB site of the NGAL promoter was required for IκB-ζ to exert its effect. The human β defensin 2 gene also required IκB-ζ for its IL-1β-specific induction in A549 cells. Our findings indicate that a common regulatory mechanism has evolved to control expression of a subset of antimicrobial proteins expressed in epithelial cells. ***The Journal of Immunology,*** **2006, 176: 5559–5566.**

Expression of a large number of genes is specifically induced or strongly increased during inflammation. The transcription factor NF-κB is a major regulator of this process. It exists as a dimeric complex composed of one or two of the five members of the NF-κB family: RelA (p65), RelB, c-Rel, NF-κB1(p50), and NF-κB2(p52) (1). In unstimulated cells, NF-κB is sequestered in the cytosol through association with a member of the IκB protein family: usually IκB-α or IκB-β (2). After activation of the cell with an appropriate stimulus, IκB-α and IκB-β are degraded, followed by a translocation of the NF-κB complex to the nucleus, where it can bind to the promoters of its target genes and induce transcription (1–3).

A number of different stimuli may lead to an inflammatory response and NF-κB activation (e.g., infection, exposure to UV light, hypoxia (1, 3, 4)). NF-κB is furthermore known to activate a large variety of genes that encode proteins with quite diverse biological functions such as antiapoptotic proteins, extracellular adhesion molecules, cytokines and chemokines, and antimicrobial proteins (1, 3). Because so many different stimuli converge in the activation of NF-κB, it is believed that some additional mechanisms must have evolved for the cell to respond appropriately to the stimuli that evoke an inflammatory response. A number of reports indicate that this fine-tuning of gene expression is achieved in part by binding of NF-κB-specific cofactors to the NF-κB complex associated with the target gene promoter and in part by coinduction of other transcription factors (e.g., through the MAPK pathway) that cooperate with the NF-κB factor on the target promoter (2, 5–8).

Recently, an IL-1β-inducible cofactor of NF-κB, named IκB-ζ (9) (also termed MAIL (10) and INAP (11)), was identified. IκB-ζ is highly homologous to Bcl-3 (9, 11), another cofactor of NF-κB belonging to the IκB family. In contrast to transcriptional repressors such as IκB-α, Bcl-3 acts as a positive activator of transcription (1). The function of IκB-ζ may be similar to that of Bcl-3, although examples of transcriptional repression by IκB-ζ have also been reported (9, 12–14). IκB-ζ is rapidly induced in response to stimulation with IL-1β and LPS, but not with TNF-α, with peak transcript levels observed 1–2 h after stimulation (9, 10, 12). When analyzing peritoneal macrophages from mice with a targeted disruption of IκB-ζ, it was found that a number of LPS-responsive genes could no longer be up-regulated (12). One of the affected genes encoded 24p3 (or lipocalin 2), which is the mouse homologue of neutrophil-gelatinase-associated lipocalin (NGAL)[3] (12).

NGAL is a 25-kDa glycoprotein, first identified as a matrix protein of specific granules of human neutrophils (15). Expression of NGAL has been observed in epithelial cells, where it is strongly induced during inflammation (16–20). NGAL belongs to the lipocalin superfamily, whose members share a barrel-shaped tertiary structure with a hydrophobic pocket that can bind lipophilic molecules (21). NGAL's ligand is bacterial ferric siderophores, which are used by bacteria for uptake of the essential nutrient iron (22, 23). Targeted disruption of the gene encoding the murine homologue of NGAL (24p3) demonstrated that the bacteriostatic effect of NGAL against a clinical strain of *Escherichia coli* measured in vitro (22) is also important in vivo (23). Expression of NGAL in epithelial cells is dependent on NF-κB, but the NGAL gene is only up-regulated by IL-1β (and LPS) and not TNF-α, even though

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[3] Abbreviations used in this paper: NGAL, neutrophil-gelatinase-associated lipocalin; β-Gal, β-galactosidase; hBD, human β defensin; IRF-3, IFN regulatory factor-3; scRNA, scrambled RNA; siRNA, small interfering RNA.

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both induce activation and promoter binding of NF-κB (12, 19, 20, 23). In this study, we demonstrate that this IL-1β specificity is caused by a requirement for the cofactor IκB-ζ (which itself is induced specifically by IL-1β and LPS) for activation of the NGAL promoter through the NF-κB signaling pathway.

Materials and Methods

Cell culture

A549 (ATCC CCL-185) cells were obtained from the American Type Culture Collection and grown in HAM F12 (Invitrogen Life Technologies) supplemented with 10% FCS (Invitrogen Life Technologies), 100 U/ml penicillin, and 100 μg/ml streptomycin (100/100 P/S) (Invitrogen Life Technologies) at 37°C in a humid atmosphere with 5% CO_2. For transfection and/or induction with IL-1β and TNF-α (both Sigma-Aldrich) or incubation with cycloheximide (Sigma-Aldrich), A549 cells were grown to 70–80% confluence and shifted to HAM F12 medium with 0.5% FCS and 100/100 P/S.

RNA isolation and Northern blot

Total RNA was prepared with TRIzol (Invitrogen Life Technologies), according to the manufacturer's recommendations, and the concentration was determined by spectrophotometric measurement. For Northern blotting, 5 μg of RNA was run on a 1% agarose gel, transferred to a Hybond-N membrane (Amersham Biosciences), and hybridized, as described (19). The membranes were washed, as described (19), and developed by a Fuji BAS2500 phosphor imager. The size of the mRNAs was determined by reference to 18S and 28S ribosomal RNA. The membranes were stripped by boiling in 0.1% SDS before rehybridization. The NGAL, IL-8, and β-actin cDNA probes have been described earlier (19). Probes for IκB-ζ and Bcl-3 were generated by PCR amplification of the entire coding region for the proteins with the following primer pairs: 1) 5′-GAAAGGACTTT GATTTGTGGC-3′ and 5′-ATCTAGTCCAATGACTGAAGC-3′, and 2) 5′-TAAAGCTTGCCACCATGGACGAGGGGCCCGTG-3′ and 5′-GAG GATCCTCAGCTGCCTCCTGGAGC-3′, respectively, using: 1) cDNA from IL-1β-stimulated A549 cells, and 2) IMAGE clone 5806689 (Bcl-3) (MRC Geneservice) as templates. The PCR products were cloned in pCR2.1-TOPO (Invitrogen Life Technologies) and verified by sequencing. The probes were radiolabeled with [α-^{32}P]dCTP using the Random Primers DNA Labeling System (Invitrogen Life Technologies). For quantitative assessments, the intensities of the IκB-ζ, NGAL, and IL-8 signals were normalized to the hybridization intensity from a probe against β-actin.

Quantitative TaqMan PCR

Quantitative PCR analysis was performed on the Applied Biosystems 7500 Real-Time PCR system (Applied Biosystems) with the commercial gene expression assay Assay-on-demand (Applied Biosystems). The assays used were: DEFB4 (gene encoding human β defensin 2 (hBD2)), Hs00175474_m1; IL-8, Hs00174103_m1; and β-actin, Hs99999903_m1.

Plasmids and small interfering RNA (siRNA)

A human IκB-ζ-specific 21-nt siRNA Silencer-predesigned siRNA (identification number 33380: sense, 5′-GGAAAAAGGUAAAUACAGCTT-3′ and antisense, 5′-GCUGUAUUUACCUUUUUCCTT-3′ (Ambion)) was used to knock down IκB-ζ mRNA. Silencer Negative Control 1 siRNA (Ambion) was included as control to analyze for any nonspecific effects of the siRNA treatment. The promoter constructs pNGP1695CAT, pNGP1695(*NF-κB*)CAT, pNGP183CAT, and pCAT3basic (Promega) are described elsewhere (19). Expression vectors for IκB-ζ and Bcl-3 were generated by excision of the coding region from pCR2.1-TOPO(IκB-ζ) and pCR2.1-TOPO(Bcl-3) described above by restriction with *Eco*RI and *Xho*I. The DNA fragments were cloned in pcDNA3.1$^+$ (Invitrogen Life Technologies) digested with the same enzymes.

Cell transfection and reporter enzyme assay

For experiments with siRNA, transfection with LipofectAMINE 2000 (Invitrogen Life Technologies) was performed according to the manufacturer's instructions. Transfections involving only plasmid DNA were performed by use of Effectene (Qiagen). For promoter studies, 0.8 μg of CAT plasmid promoter construct was cotransfected with 0.2 μg of pcDNA3-β-Gal (encoding β-galactosidase (β-Gal)) to compensate for differences in transfection efficiency. Expression of the reporter enzymes was quantitated by CAT and β-Gal ELISA (Roche Diagnostic Systems), according to the manufacturer's recommendations. For each sample, the CAT activity was normalized to β-Gal activity.

Quantitation of NGAL, IL-1β, and IL-8 in medium

NGAL was quantitated by ELISA, as described previously (24). IL-1β and IL-8 were quantitated with the IL-1β and IL-8 optEIA ELISA kits (BD Pharmingen), according to the manufacturer's recommendations.

SDS-PAGE and immunoblotting

SDS-PAGE and immunoblotting were performed according to the instructions given by the manufacturer (Bio-Rad). For immunodetection, the polyvinylidene fluoride membranes (Millipore) were blocked for 1 h with 5% skimmed milk in PBS after the transfer of proteins from 14% polyacrylamide gels, and incubated overnight with rabbit anti-IκB-ζ Abs (12) (1:1000) and anti-α-tubulin (Sigma-Aldrich; T-9026) (1:4500). The following day, the membranes were incubated for 2 h with peroxidase-conjugated porcine anti-rabbit Abs (DakoCytomation; P-0217) and visualized ECL (Amersham Biosciences).

Results

IκB-ζ is specifically up-regulated by IL-1β in A549 cells

We have reported earlier that NGAL is up-regulated by IL-1β, but not by TNF-α, in human epithelial cells in an NF-κB-dependent manner, despite identical ability of both cytokines to induce binding of NF-κB to the NGAL promoter (19). It was recently reported that a nuclear binding partner of NF-κB (named IκB-ζ) is selectively induced by IL-1β in a number of tissues (9). Furthermore, it was reported that IκB-ζ could act both as an activator and a repressor of NF-κB-dependent transcription (9, 12–14). To investigate whether IκB-ζ could be responsible for the IL-1β-specific up-regulation of NGAL, we analyzed the transcript profile of IκB-ζ in the lung epithelial cell line A549 that we have previously used as model cell line (19). Strong induction of IκB-ζ was observed in IL-1β-stimulated cells with peak transcript level at 1.5 h (Fig. 1). No IκB-ζ mRNA was observed in uninduced cells, and only weak induction was seen following administration of TNF-α. In contrast, the mRNA level of the closely related cofactor Bcl-3 (9, 11), which has also been associated with transcriptional modulation of NF-κB (1, 5), was unaltered following stimulation with each of these cytokines (Fig. 1).

De novo protein synthesis is required for IL-1β induction of NGAL synthesis

These data indicated that IκB-ζ could function as a positive activator of NF-κB on the NGAL promoter. To determine whether a newly synthesized cofactor was required for activation of the NGAL promoter, we stimulated A549 cells with IL-1β in the presence of cycloheximide, which blocks new protein synthesis. Inclusion of cycloheximide abrogated the strong induction of NGAL

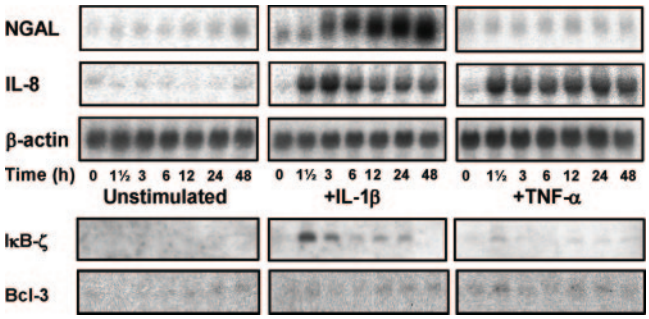

FIGURE 1. Kinetics of IL-1β-specific induction of NGAL and IκB-ζ transcripts in A549 cells. Cells were harvested at the indicated time points after addition of fresh medium (unstimulated) or medium supplemented with IL-1β (100 pg/ml) or TNF-α (20 ng/ml). RNA was isolated and hybridized to ^{32}P-labeled probes, as indicated. The data are representative of two independent experiments. The *upper part* of the blot has previously been published in the *Journal of Immunology* (19) (copyright 2003, American Association of Immunologists).

mRNA normally seen 4–6 h after administration of IL-1β (Figs. 1 and 2). In contrast, induction of the transcripts for IL-8 and IκB-ζ was unaffected by the presence of cycloheximide; in fact, an increase in mRNA levels was observed for the latter two targets, indicating a stabilization of these two transcripts under these circumstances. Taken together, this demonstrated a need for de novo protein synthesis for IL-1β induction of the NGAL promoter. It is further seen in Fig. 3*C* that IL-1β induces an accumulation of IκB-ζ in A549 cells.

Inhibition of IκB-ζ expression results in decreased NGAL production

To further validate that IκB-ζ could be the cofactor required for NGAL induction by IL-1β, we transfected A549 cells with a specific siRNA against IκB-ζ. Northern blot analysis of a sample collected 2 h after IL-1β stimulation demonstrated a 3-fold reduction of IκB-ζ mRNA in the presence of IκB-ζ-siRNA, whereas addition of scrambled RNA (scRNA), which was used as control for the siRNA, did not down-regulate IκB-ζ mRNA levels (Fig. 3). The IL-8 mRNA also peaked 2–3 h following IL-1β stimulation (Figs. 1 and 2), but the latter transcript was unaffected by the presence of both scRNA and siRNA, demonstrating the specificity of the IκB-ζ-siRNA (Fig. 3). Following IL-1β stimulation, NGAL mRNA levels accumulate for 24–48 h (Fig. 1). We therefore decided to examine the effect of IκB-ζ-siRNA on NGAL mRNA levels after 24 h. The level of NGAL mRNA was greatly diminished in IκB-ζ-siRNA-treated cells compared with untreated or scRNA-treated cells. Again, no difference was seen for IL-8 mRNA levels between scRNA- and siRNA-treated cells. This indicates that the IκB-ζ cofactor remains active for a long period when first synthesized (11).

To determine the effect of the siRNA on the proteins encoded by the above-mentioned mRNAs, we made another experiment in which endogenous IκB-ζ protein levels were measured 2 h after IL-1β stimulation by Western blot of whole cell lysates. As was the case for the cognate transcript, the level of the IκB-ζ cofactor was greatly diminished in siRNA-treated cells (Fig. 3*C*). Although the level of IL-8 mRNA peaked after 2–3 h, a continuous accumulation of both IL-8 and NGAL protein in the cell culture medium occurred during the entire 24-h IL-1β stimulation period (19). For this reason, the content of NGAL and IL-8 in culture medium was measured at 24 h. The amount of NGAL in the medium of IκB-ζ-siRNA-treated cells followed the same pattern as observed for the NGAL mRNA (Fig. 3, *B* and *D*). For IL-8, the amount of protein synthesized was marginally affected by the presence of either scRNA or siRNA (Fig. 3*D*).

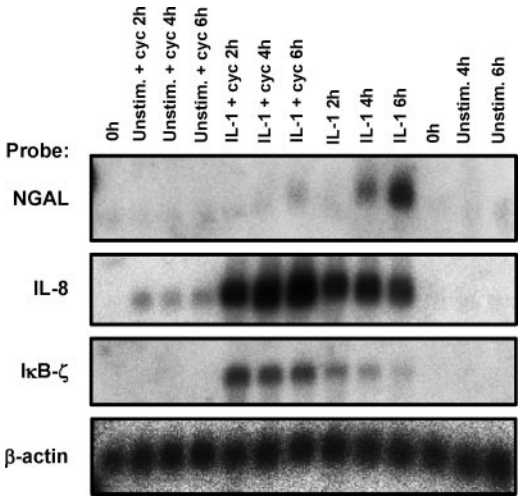

FIGURE 2. IL-1β induction of NGAL mRNA synthesis requires de novo protein synthesis. Cells were harvested at the indicated time points after addition of fresh medium (unstim.) or medium supplemented with IL-1β (100 pg/ml) in the presence or absence of 10 μg/ml cycloheximide (cyc), which prevents protein synthesis. RNA was isolated and hybridized to ^{32}P-labeled NGAL, IL-8, IκB-ζ, and β-actin cDNA probes. The amount of IL-8 and IκB-ζ mRNA was higher in cells coincubated with cycloheximide than in cells receiving normal medium, indicating that a protein/enzyme that usually destabilizes these transcripts was not produced in the former case.

Coexpression of IκB-ζ enables NGAL promoter activation by TNF-α stimulation

We have demonstrated previously that both TNF-α and IL-1β stimulation can induce binding of NF-κB to the NGAL promoter, but up-regulation of NGAL promoter activity was only observed after stimulation with IL-1β (19). To validate that the coinduction of IκB-ζ by IL-1β can explain the IL-1β specificity of the NGAL promoter, we cotransfected A549 cells with an NGAL promoter construct and an expression plasmid encoding IκB-ζ and subsequently stimulated with TNF-α to induce activation of NF-κB. A slight induction was observed when A549 cells transfected with control vector were stimulated with TNF-α, as observed earlier (19). In contrast, a 6- to 8-fold increase in NGAL promoter activity was seen in TNF-α-stimulated cells cotransfected with the IκB-ζ-expressing vector (Fig. 4*A*). Increasing the amount of cotransfected IκB-ζ-expressing vector caused a concomitant increase in the NGAL promoter activity, which was not the case when the amount of control vector was increased in a similar manner (Fig. 4*B*). Cotransfection with a Bcl-3-expressing vector did not have any effect on NGAL promoter activity following TNF-α stimulation (Fig. 4*A*). Taken together, these data demonstrate that expression of IκB-ζ is sufficient to transform TNF-α into a potent inducer of NGAL promoter activity. This indicates that it is the ability of IL-1β to induce both IκB-ζ expression and NF-κB activation that allow it to up-regulate NGAL promoter activity in contrast to TNF-α, which does not induce IκB-ζ expression.

An intact NF-κB binding site is required for IκB-ζ-mediated induction of the NGAL promoter

The NF-κB binding site at position -180 to -171 has been shown to be required for IL-1β induction of the NGAL promoter (19). To determine whether the effect of IκB-ζ could be ascribed to an interaction between IκB-ζ and the p65:p50 NF-κB heterodimer binding to this κB element of the NGAL promoter, we examined the consequence of IκB-ζ-siRNA on cells transfected with a 1695-bp NGAL promoter construct with either the wild-type κB-binding sequence (pNGP1695) or a mutant of this promoter (pNGP1695M) in which the ($-180/-171$) κB element is unable to bind the p65:p50 NF-κB factor (19). As expected, siRNA against IκB-ζ caused 8- to 9-fold reduction of wild-type NGAL promoter activity following IL-1β induction compared with the control with scRNA (Fig. 5*A*). A small reduction of promoter activity by IκB-ζ-siRNA compared with scRNA was also observed in uninduced cells for both the 1695-bp promoter and the 5′ deletion mutant pNGP183, which terminate 3 bases upstream of the $-180/-171$ NF-κB site, indicating slight activation of the NGAL promoter by NF-κB:IκB-ζ under these circumstances. In contrast, the activities of the pNGP1695M promoter in IκB-ζ-siRNA- and scRNA-treated cells were comparable: both in uninduced and IL-1β-stimulated cells. Although the activity of pNGP1695M only increased 2- to 3-fold when stimulated by IL-1β (Fig. 5*B*), this activation did not appear to be dependent on IκB-ζ. Taken together, these data indicate that

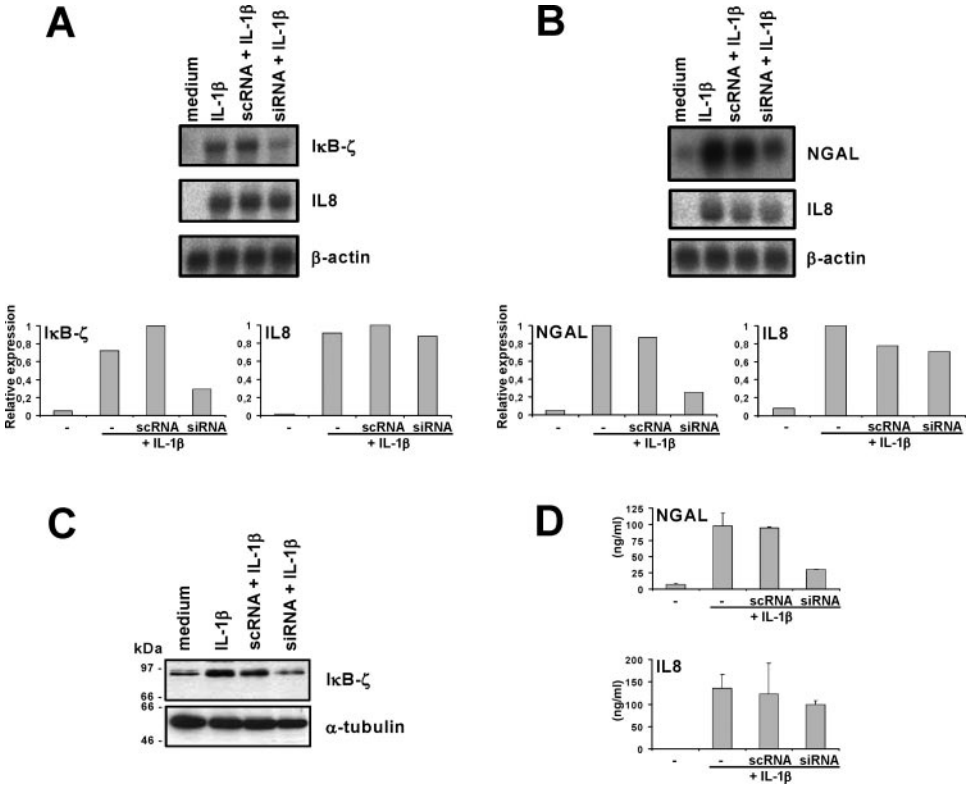

FIGURE 3. Down-regulation of IκB-ζ expression causes a decrease of NGAL expression. *A* and *B*, Untransfected A549 cells (−) or cells transfected with 40 nmol control-siRNA (scRNA) or IκB-ζ-siRNA (siRNA) were grown in medium without or with 100 pg/ml IL-1β for 2 h (*A*) or 24 h (*B*), and then harvested for RNA isolation. RNA was hybridized to ^{32}P-labeled probes for *A*, IκB-ζ and IL-8, and *B*, NGAL and IL-8. Hybridization to β-actin was performed to assure equal loading. Relative expression levels (after normalization to β-actin) are shown as diagrams below the blots: the expression levels are shown relative to that of IL-1β-stimulated cells transfected with scRNA (value = 1). *C*, Protein (whole cell lysates) was purified from cells transfected and treated as in *A* and analyzed for IκB-ζ by Western blot. A blot against α-tubulin was included to assure equal loading. *D*, Medium was collected from cells transfected and treated as in *B* and analyzed for NGAL and IL-8 by ELISA. One of three independent experiments is shown in *A–C*.

IκB-ζ only interacts with the NF-κB complex bound to the −180/−171 site, as neither basal nor the residual IL-1β-induced expression of a promoter without a functional NF-κB element was affected. As discussed later, such interaction cannot be visualized by EMSA because the oligonucleotides used for this do not encompass both the NF-κB binding site and the sequence information required for IκB-ζ binding.

IL-1β stimulation is essential for induction of NGAL synthesis, but can be partially compensated by TNF-α stimulation at later stages

NGAL accumulated in the medium of IL-1β-stimulated A549 cells during the entire 48-h induction period, whereas no increase was observed for TNF-α-stimulated cells compared with uninduced cells, not even at the end of the experiment. TNF-α is known to induce a rapid de novo production of a number of cytokines, as exemplified by IL-8 (Figs. 1 and 6). If TNF-α likewise induced IL-1β production in A549 cells, a delayed, but measurable induction of NGAL synthesis by this newly synthesized IL-1β would be expected. To determine whether TNF-α induced IL-1β synthesis, we measured IL-1β levels at different time points following stimulation. As demonstrated in Fig. 6*A*, no increase in IL-1β levels was observed in medium from TNF-α- or uninduced cells. Furthermore, a high level of IL-1β was still present in the medium of IL-1β-stimulated cells at the end of the experiment, probably explaining how NGAL and IL-8 synthesis could be sustained for 48 h.

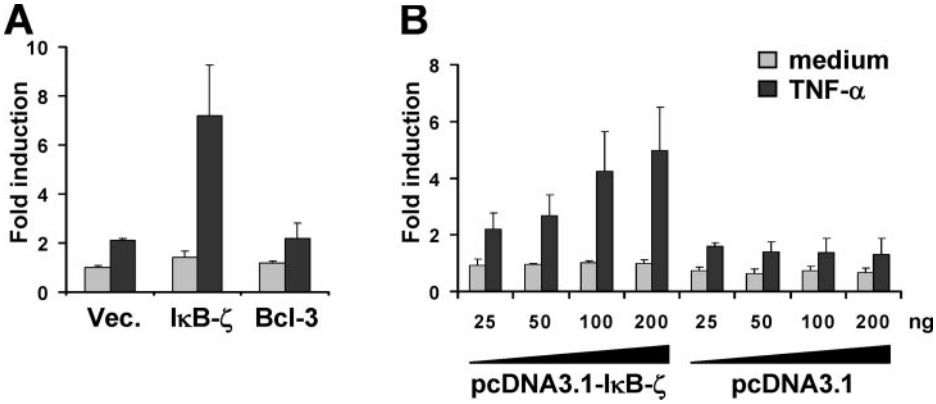

FIGURE 4. The NGAL promoter can be up-regulated by TNF-α if IκB-ζ is constitutively expressed and requires a functional NF-κB element. *A*, A549 cells were cotransfected with a CAT reporter plasmid carrying a 1695-bp fragment of the wild-type NGAL promoter (pNGP1695CAT, 0.7 μg) and 0.2 μg of either an empty expression vector (Vec.), or a vector expressing IκB-ζ or Bcl-3. After 24 h in growth medium or medium supplemented with 20 ng/ml TNF-α, the cells were harvested and promoter activities were determined. CAT activities are shown relative to that of the unstimulated empty CAT reporter (value = 1). *B*, A549 cells were cotransfected with pNGP1695CAT (0.7 μg) and 25–200 ng of either a vector expressing IκB-ζ (pcDNA3.1-IκB-ζ) or the empty expression vector (pcDNA3.1). All results are the mean ± SD of three independent transfections. In all cases, the CAT activity was normalized to the β-Gal activity from the cotransfected vector pcDNA3-β-Gal (0.2 μg).

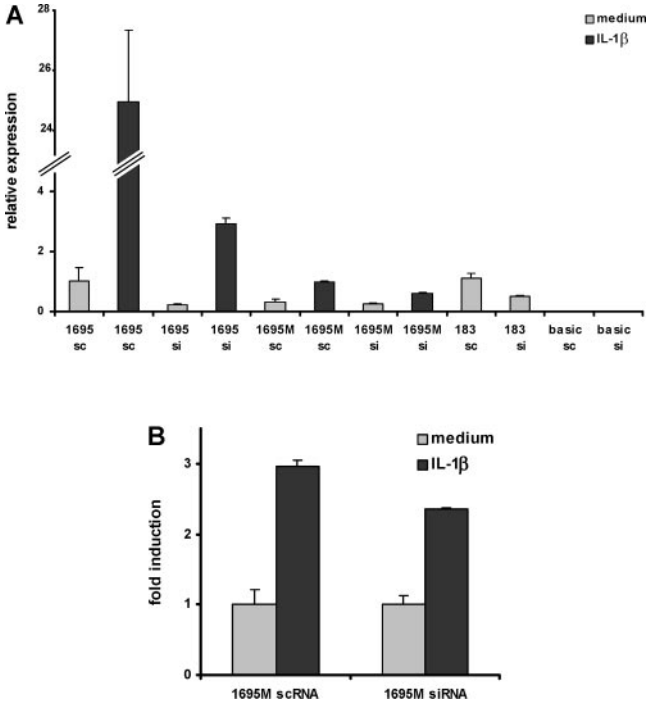

FIGURE 5. Down-regulation of NGAL promoter activity by IκB-ζ-siRNA requires a functional NF-κB binding site. *A*, A549 cells were transfected with a CAT reporter plasmid carrying: 1) a 1695-bp fragment of the wild-type NGAL promoter (1695); 2) a NF-κB-mutated NGAL promoter (1695M); 3) a deletion mutant terminating 183 bp upstream of Cap-site (183); or 4) no promoter fragment (basic) and either control-siRNA (scRNA) or IκB-ζ-siRNA (siRNA). After 24 h in growth medium (medium) or medium supplemented with 100 pg/ml IL-1β, the cells were harvested and promoter activities were determined. CAT activities are shown relative to that of the unstimulated 1695 CAT reporter receiving scRNA (value = 1). *B*, Fold induction of 1695M after IL-1β stimulation relative to the expression of the 1695M promoter in unstimulated cells. All results are the mean ± SD of three independent transfections. In all cases, the CAT activity was normalized to the β-gal activity from the cotransfected vector pcDNA3-βGal.

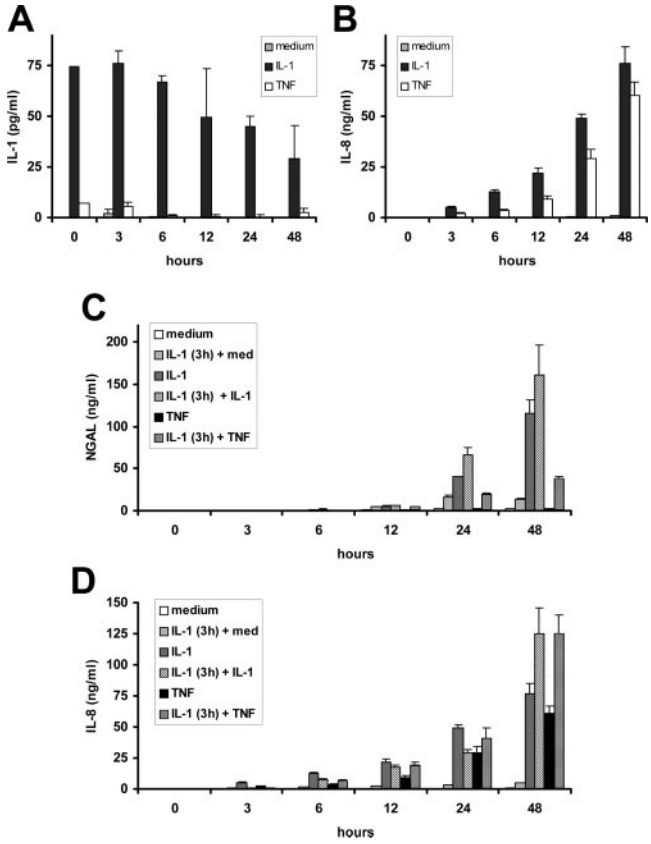

FIGURE 6. Measurement of IL-1β, IL-8, and TNF-α concentrations in medium from A549 cells stimulated with inflammatory mediators. The amount of IL-1β (*A*) and IL-8 (*B*) was determined in medium of A549 cells retrieved at the indicated time points after addition of fresh medium (medium) or medium supplemented with IL-1β (100 pg/ml) or TNF-α (20 ng/ml). The amount of NGAL (*C*) and IL-8 (*D*) was determined at the indicated time points in medium of A549 cells prestimulated with IL-1β (100 pg/ml) for 3 h and then changed to fresh medium (medium) or medium supplemented with IL-1β (100 pg/ml) or TNF-α (20 ng/ml) for the remaining 45 h. Medium from cells incubated under the same conditions without prestimulation was also analyzed. The concentrations of IL-1β, IL-8, and NGAL are shown as the mean ± SD of three independent experiments. The experiment was performed twice with similar results.

Although the data clearly demonstrate that NGAL is induced specifically by IL-1β, it is possible that this stimulus is required only during formation of an active transcription complex composed of NF-κB and IκB-ζ. Because expression of IκB-ζ peaks after 1.5–2 h (Figs. 1 and 2), it is feasible that further stimulation with IL-1β is required only to maintain NF-κB in an active state. If this was the case, stimulation with IL-1β for 3 h followed by stimulation with TNF-α should result in an NGAL synthesis similar to that obtained with IL-1β alone. As demonstrated in Fig. 6*C*, increased NGAL production was observed in cells prestimulated with IL-1β for 3 h before TNF-α stimulation, compared with cell stimulated with TNF-α alone. The amount of NGAL synthesized was 32 and 23% of that produced by cells stimulated with IL-1β for 48 h and 3 + 45 h, respectively. However, a higher NGAL expression was also observed in cells prestimulated 3 h with IL-1β and then changed to medium with or without IL-1β compared with cells growing under the same conditions without prestimulation. Likewise, a significant increase in IL-8 synthesis was observed under all three growth conditions for cells prestimulated with IL-1β. Taken together, these data indicate that with regard to NGAL synthesis, TNF-α can compensate to some degree for the signal generated by IL-1β from 3 h and beyond.

IL-1β-specific up-regulation of hBD2 is also dependent upon IκB-ζ expression

In an earlier publication, we argued that the IL-1β-specific up-regulation of the antimicrobial protein NGAL might in fact reflect an adaptation to the TLR pathway, which uses the same intracellular signaling pathway as the IL-1R (19). This was verified by demonstrating that induction of NGAL promoter activity could be achieved through TLR-4 by the bacterial ligand LPS (19). If the IL-1β specificity does reflect such an adaptation, one could expect that genes for other antimicrobial proteins would be regulated in the same manner as the NGAL gene, i.e., requires the induction of both IκB-ζ and NF-κB for transcriptional activation. Recently, we demonstrated that both NGAL and hBD2 were up-regulated by IL-1β, but not by TNF-α or IL-6 in human keratinocytes (19, 20). To determine whether the same specificity applied for A549 cells, we first tested whether hBD2 was also specifically up-regulated by IL-1β in these cells and found that this was indeed the case (Fig. 7*A*). hDB2 transcripts accumulate during the entire period of stimulation (20), and hBD2 mRNA levels were therefore measured 24 h poststimulation in A549 cells, similar to NGAL transcripts.

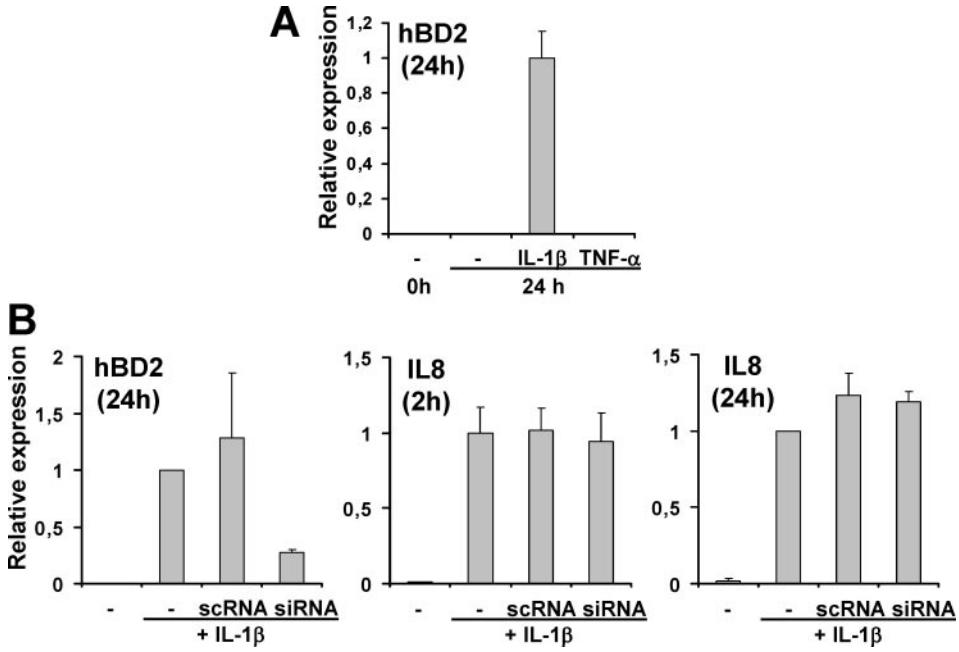

FIGURE 7. Down-regulation of IκB-ζ expression causes a decrease of hBD2 expression. *A*, RNA from unstimulated A549 cells (−) or cells stimulated with IL-1β (100 pg/ml) or TNF-α (20 ng/ml) for 0 and 24 h was analyzed for hBD2 expression by real-time PCR. Only following IL-1β treatment was the hBD2 transcript detectable. *B*, A549 cells transfected with 40 nmol control-siRNA (scRNA) or IκB-ζ-siRNA (siRNA) were grown in medium without or with 100 pg/ml IL-1β for 2 or 24 h, as indicated, and then harvested for RNA isolation. The amount of hBD2 and IL8 transcript was determined by real-time PCR relative to the expression of β-actin. Relative expression levels (after normalization to β-actin) are shown as diagrams below the blots: the expression levels are shown relative to that of IL-1β-stimulated cells transfected with scRNA (value = 1).

Next, we analyzed the effect of treating the cells with IκB-ζ-siRNA. At 24 h of IL-1β stimulation, the level of hBD2 transcript in IκB-ζ-siRNA-treated cells was <20% of that measured in cells not receiving external RNA- and scRNA-treated cells. As control, we measured the amount of IL-8 mRNA 2 and 24 h after stimulation with IL-1β and found, as before, no effect of either siRNA or scRNA on IL-8 transcript levels (Fig. 7*B*). This indicates that a common regulatory mechanism exists for the two antimicrobial proteins NGAL and hBD2.

Discussion

Induction of inflammatory response genes by the NF-κB pathway is more complex than just the mere binding of an NF-κB factor to the κB site(s) of NF-κB-responsive genes. Involvement of NF-κB-binding cofactors and NF-κB-interacting transcription factors such as C/EBP, c-*jun*, or AP-1 induced by the MAPK pathway (6, 7) has added an additional layer of complexity to the regulatory mechanism used by cells to tailor their response to the biological condition causing the inflammatory response. A subset of NF-κB target genes encodes antimicrobial proteins such as NGAL and hBD2, and these should only be induced during a microbial challenge. In this study, we present evidence that the specific induction of these two innate immune defense proteins is critically dependent on induction of the NF-κB cofactor IκB-ζ.

We show in this study that the IL-1β selectivity in inducing NGAL and hBD2 expression in epithelia can be explained by the synthesis of IκB-ζ elicited specifically by IL-1β stimulation. Activation of NGAL requires de novo protein synthesis (Fig. 2) and is strongly diminished when the IκB-ζ transcript is destabilized by siRNA (Fig. 3). An effect of the IκB-ζ-siRNA on the NGAL promoter was, however, only observed provided a functional κB element at position −180/−171 was present (Fig. 5). If NF-κB was unable to bind this κB site, the IκB-ζ-siRNA had no further effect on the NGAL promoter, indicating that IκB-ζ did not bind the NGAL promoter by itself nor interacted with an NF-κB factor binding to another κB element of NGAL promoter. The observation that forced expression of IκB-ζ could rescue the inability of TNF-α to induce NGAL promoter activity strongly indicates that the feature that discriminates between nonactivation of the NGAL promoter by TNF-α and activation by IL-1β is the ability of the latter cytokine to induce synthesis of IκB-ζ in addition to activating NF-κB (Fig. 4).

The explanation for the IL-1β specificity of IκB-ζ expression is due to stabilization of the IκB-ζ mRNA when stimulating with IL-1β (and other ligands using the IL-1R signaling pathway such as LPS activation through TLR-4 (12)). Induction of IκB-ζ gene expression depends on activation of NF-κB, but efficient synthesis of IκB-ζ furthermore requires stabilization of the transcript (14). TNF-α stimulation does not result in stabilization of the IκB-ζ transcript. Stimulation by TNF-α, therefore, does not result in increase of the IκB-ζ protein level despite its ability to induce IκB-ζ gene expression through NF-κB activation (13). The signaling pathways leading to activation of NF-κB and stabilization of the IκB-ζ mRNA thus appear to be separate. This notion is strengthened by the observation that stimulation with IL-17 causes a stabilization of the IκB-ζ transcript without activating NF-κB (14). In accordance with the results shown in this study (Fig. 4), costimulation with TNF-α and IL-17 is able to cause a 7- to 8-fold induction of the murine homologue of NGAL (lcn-2/24p3) in the preosteoblast cell line MC3T3-E1 compared with the combined stimulatory effect of TNF-α and IL-17 by themselves (25). Analysis of the 24p3 promoter in MC3T3-E1 cells showed a 1.7- and 2.8-fold induction, respectively, by IL-17 and TNF-α alone, and a 22.6-fold induction of promoter activity by IL-17 plus TNF-α (25). In contrast, the combined effect of TNF-α stimulation and constitutive coexpression of IκB-ζ demonstrated in this study resulted only in a 7- to 8-fold increase of promoter activity in A549 cells (Fig. 4). This, however, can be explained by the lack of an IκB-ζ-mRNA-stabilizing signal under these experimental conditions compared with stimulation with IL-1β or IL-17. The *cis*-element causing destabilization of the IκB-ζ mRNA is contained within the open reading frame of the transcript (14), and thus is present in the expression construct used by us.

In addition to the in vitro data, a requirement of IκB-ζ for in vivo up-regulation of 24p3 (the murine homologue of NGAL) through the IL-1R/TLR-4 signaling pathway has recently been demonstrated by the lack of 24p3 expression in peritoneal macrophages from IκB-$\zeta^{-/-}$ mice after LPS stimulation (12). This contrasts with the strong up-regulation of the 24p3 transcript seen in wild-type mice (12). Taken together, these data strongly indicate

that the IL-1β specificity of the NGAL promoter is governed by IκB-ζ.

Which feature of the NGAL promoter determines the requirement for the cofactor IκB-ζ? Comparisons of the κB sites of genes that need IκB-ζ for induction through the IL-1R/TLR pathway show no obvious common pattern (12). Analysis for subunit specificity of the individual proteins of the dimeric NF-κB factor likewise failed to identify any common characteristics in the examined κB sites that could explain the binding of different homo- and heterodimers of NF-κB (26). In fact, the sequence of the κB site of NGAL (GGGAATGTCC) is also found in the promoter of the IκB-ζ gene, but in this case mutation of the κB site had no effect on the promoter activity of the IκB-ζ gene (27, 28). A recent publication demonstrated that a single base difference in the κB site of the MCP-1 gene was sufficient to alter the cofactor specificity of the NF-κB molecule bound to this site from IFN regulatory factor-3 (IRF-3) to Bcl-3 (5). The authors speculated that cofactor specificity was determined by a specific structural conformation of the NF-κB dimer, imposed upon the protein complex by the DNA sequence it bound to. A similar mechanism could determine the specificity of the p65:p50 dimer binding to the NGAL promoter for the cofactor IκB-ζ, as altering the sequence of the κB element of the NGAL promoter to that of the IL-8 promoter is sufficient to abolished IL-1β induction of NGAL (19). In contrast, the sequence of the NGAL κB element (and the five flanking bases on each side) was not sufficient to impose IL-1β specificity to a heterologous SV40 promoter (19). Increasing the number of flanking bases on each side of the NGAL κB element to 20 likewise had no effect (data not shown), indicating, as suggested previously, that a second transcription factor binding 100–150 bases downstream of the NGAL ($-180/-171$) κB site is also required for IL-1β responsiveness of the NGAL gene (19). This may explain why identical band patterns were observed in an EMSA with the NGAL κB element following IL-1β and TNF-α stimulation (19) (data not shown) because the sequence information for binding of IκB-ζ was probably not contained within the oligos used for the experiment.

A requirement for interaction with two nonadjacent transcription factors for cofactor binding to NF-κB has been described for the MCP-1 and IP-10 promoters. In this case, two κB sites were needed for NF-κB to bind the cofactors IRF-3 and Bcl-3 (5). A second potential κB site exists at $-91/-82$ of the NGAL promoter, but as mutation of this sequence influences neither promoter activity nor specificity (J.B. Cowland and N. Borregaard, manuscript in preparation), we do not believe that this DNA element regulates IκB-ζ binding.

The IκB-ζ mRNA level peaks 1.5–2 h after IL-1β stimulation and then rapidly declines (Fig. 1). This indicates that IκB-ζ synthesis is required only during assembly of a transcription complex on the NGAL promoter, and that continued IL-1β stimuli is needed to keep the NF-κB complex associated with the NGAL promoter. This notion is supported by the observation that TNF-α stimulation to some degree compensates for the signal generated by IL-1β, as cells stimulated for 3 h with IL-1β and then with TNF-α for 45 h produced 25–30% of the NGAL measured for cells stimulated with IL-1β for all 48 h (Fig. 6*C*). However, the level of NGAL synthesized by cells pretreated with IL-1β (3 h) and then changed to medium with TNF-α (45 h) was only three times that produced by cells that were changed to medium without cytokines. This indicates that shortly after formation of the transcription complex, an inactivation phase follows, which is impeded partly by TNF-α stimulation and efficiently by IL-1β stimulation.

Following activation of NF-κB, a number of genes are induced, including that encoding IκB-α (3, 29). This causes a de novo production of IκB-α that can interact with NF-κB complexes bound to DNA and mediate their transport back to the cytosol (3). This negative feedback loop will in many cases terminate NF-κB-induced transcription. NF-κB may, however, also interact with the related factor IκB-β. Stimulation with, for example, IL-1β also results in degradation of IκB-β, although with a slower kinetic than for IκB-α (29, 30). De novo synthesis of IκB-β is also induced, but in this case an unphosphorylated form is generated that can interact with DNA-bound NF-κB and act as a chaperone that hinders binding of, and inactivation by, IκB-α (30, 31). This may cause persistent activation of the promoter by NF-κB rather than rapid inactivation by IκB-α. Further stabilization of the DNA-bound NF-κB complex can be obtained by acetylation of the p65 subunit that also blocks for binding of IκB-α (32). In cases in which IκB-ζ is involved (as for the NGAL promoter), a further level of complexity is introduced because IκB-ζ can act both as an activator and repressor of NF-κB-mediated transcription (13). It has been demonstrated that IκB-ζ binds to the p50 subunit in a manner analogous to that of Bcl-3, but its mechanism of action is unknown (9).

Based on this information, the following model can explain the findings of this study: continued expression of NGAL during IL-1β stimulation is obtained by binding of IκB-β to the NF-κB:IκB-ζ complex or by an exchange of IκB-ζ with IκB-β. The stability of this complex is challenged by the newly synthesized IκB-α that will try to displace IκB-β and/or IκB-ζ from NF-κB. Continued stimulation with IL-1β retains a high level of IκB-β, and disassembly of the active transcription complex occurs slowly. Stimulation with IL-1β for 3 h allows the assembly of the IκB-ζ:NF-κB:DNA complex. When the cells subsequently are exposed to fresh medium with TNF-α, this cytokine will cause degradation of the newly synthesized IκB-α and thus delay disassembly of the NF-κB:DNA complex. Conversely, a faster down-regulation of NGAL synthesis occurs when the cells receive fresh medium without cytokines, as IκB-α remains stable under these circumstances.

As alluded to before, there is a need for specificity and selectivity of the responses elicited by NF-κB activation. This may be the reason that the genes encoding NGAL and hBD2 require IκB-ζ for activation, as this cofactor is induced by a number of bacterial TLR ligands (12). Other antimicrobial proteins such as human cationic antimicrobial protein of 18 kDa, hBD1, hBD3, and secretory leukocyte protease inhibitor are regulated in a different manner that does not in all cases involve the NF-κB pathway (20), and therefore might reflect an adaptation of the cell to antimicrobial challenges that are recognized by other mechanisms than through the TLR system. Many of the NF-κB target genes, in contrast, do not encode antimicrobial proteins, but rather antiapoptotic proteins, extracellular adhesion molecules, cytokines, or chemokines (1, 3). In the case of an aseptic inflammatory stimulus (e.g., UV radiation and hypoxia) (1, 4), it is plausible that IκB-ζ is not induced and that NF-κB instead interacts with cofactors such as Bcl-3, IRF-3, IκB-β, or IκB-ϵ (2, 5) and/or other transcription factors (6–8). This would probably lead to the activation of a different subset of NF-κB-responsive genes than those observed during infection. Further knowledge about the stimuli that regulate the association between NF-κB and its different cofactors may give us the answer as to how the NF-κB pathway adjusts to the many different biological functions it governs.

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Disclosures

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