ReLB Sustains IkBa Expression during Endotoxin Tolerance

Xiaoping Chen,1* Barbara K. Yoza,1,2 Mohamed El Gazzar,1 Jean Y. Q. Hu,1 Sue L. Cousart,1 and Charles E. McCall1

Department of Internal Medicine, Section of Molecular Medicine,1 and Department of General Surgery,2
Wake Forest University School of Medicine, Medical Center Boulevard, Winston-Salem, North Carolina 27157

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Transcription factors and chromatin structural modifiers induce clinically relevant epigenetic modifications of blood leukocytes during severe systemic inflammation (SSI) in humans and animals. These changes affect genes with distinct functions, as exemplified by the silencing of a set of acute proinflammatory genes and the sustained expression of a group of antimicrobial and anti-inflammatory genes. This paradigm is closely mimicked in the THP-1 human monocyte cell model of lipopolysaccharide (LPS) endotoxin tolerance. We previously reported that LPS-induced de novo expression of ReLB is required for generating tolerance to interleukin-1B (IL-1B) and tumor necrosis factor alpha (TNF-α) expression. ReLB represses transcription by binding with heterochromatin protein 1 α (HP1α) to the proximal promoters of IL-1β and TNF-α. In contrast, we report herein that ReLB is required for sustained expression of anti-inflammatory IkBa in LPS-tolerant THP-1 cells. ReLB transcription activation requires binding to the IkBa proximal promoter along with NF-κB p50 and is associated with an apparent dimer exchange with p65. We also observed that ReLB induced during human SSI binds to the IkBa proximal promoter of circulating leukocytes. We conclude that ReLB functions as a dual transcription regulator during LPS tolerance and human SSI by activating and repressing innate immunity genes.

Alterations in gene expression occur in circulating and tissue monocytes and neutrophils during severe systemic inflammation (SSI), such as sepsis, and in cell models of SSI, such as lipopolysaccharide (LPS)-mediated tolerance in THP-1 monocytes (30). These genes have distinct functions, including persistent repression of a set of acute proinflammatory genes that initiate SSI and sustained expression of a group of anti-inflammatory and antimicrobial genes (7, 10, 17, 28).

The formation of the transcriptionally active NF-κB heterodimer on cognate DNA sequences plays a crucial role in initiating the expression of many innate immunity genes with acute proinflammatory functions that participate in SSI (24). We reported that disruption of the NF-κB p65/p50 heterodimer formation at the proximal interleukin-1β (IL-1β) and tumor necrosis factor alpha (TNF-α) promoters occurs during epigenetic transcription repression of these genes in LPS tolerance and in human SSI (6, 9). We also discovered that de novo LPS induction of NF-κB transcription factor ReLB is required for sustaining repression of IL-1β and TNF-α transcription in the THP-1 cell model of LPS tolerance (31). The repressor effect of ReLB is coupled to the heterochromatin silencing marks of dimethylation of histone H3 lysine 9 and heterochromatin protein 1 α (HP1α) binding (9). This epigenetic repressor profile also occurs in LPS-tolerant circulating leukocytes obtained from humans with SSI (31).

The mechanisms controlling the sustained expression of anti-inflammatory genes during endotoxin tolerance are unclear. One such critically important immune modulator is IkBa.

IkBa has the most efficient inhibitory effect on NF-κB proteins (IkBa, IκBβ, IκBγ, and BCL-3) (2, 25, 27). Unlike NF-κB transcription factors, IkBa is synthesized and degraded very rapidly in the basal and stimulated states, where it dynamically exists in free and bound states, respectively (1, 13). IkBa acts as an anti-inflammatory mediator by binding to NF-κB factors p65 and p50 and sequestering them primarily in the cytosol (21). Other NF-κB dimers, such as ReLB and p52, do not tightly interact with IkBa. Stimulation by LPS results in rapid IkBa phosphorylation and proteosomal degradation, allowing the NF-κB p65/p50 heterodimer to translocate into the nucleus and activate the transcription of a large set of target genes, including a positive feedback signal from IkBa (14). The crucial role of IkBa in regulating inflammation is supported by the observation that either the genetic ablation of IkBa in mice generates SSI or the repression of IkBa degradation is immunosuppressive (11, 26). We previously reported that rapid and sustained resynthesis of IkBa contributes to the repressive signature associated with LPS tolerance in the THP-1 human monocyctic cell model of SSI (15).

The objective of this study was to determine whether ReLB might differentially regulate gene transcription during LPS tolerance of THP-1 cells and in human SSI. We found that ReLB functions in LPS tolerance of THP-1 monocytes as an essential transcription activator at the core promoter of the IkBa gene, thus contributing to sustaining the expression of this important anti-inflammatory mediator. This process involves a dimer exchange at the IkBa core promoter, with the replacement of p65/p50 by ReLB/p50 after ReLB is induced by LPS. We also observed that ReLB binds to the IkBa proximal promoter in circulating leukocytes obtained from humans with SSI. We conclude that ReLB, following its induction by LPS or during SSI, acts on specific genes and is an indispensable transcription regulator with dual functions, acting both as a transcription

* Corresponding author. Mailing address: Department of Internal Medicine, Section of Molecular Medicine, Wake Forest University School of Medicine, Medical Center Boulevard, Winston-Salem, NC 27157. Phone: (336) 716-4377, Fax: (336) 716-1214. E-mail: xchen@wfubmc.edu.

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activator and as a repressor in the same cell type, thereby influencing the epigenetic profile of innate immunity.

**MATERIALS AND METHODS**

**Cell culture and stimulation.** THP-1 cells obtained from the American Type Culture Collection were maintained in RPMI 1640 medium ( Invitrogen Life Technologies) supplemented with 10% Fetal calf serum, 2 mM l-glutamine, and 10% fetal bovine serum (HyClone) at 37°C and 5% CO₂ in a humidified incubator. LPS-mediated tolerance that mimics the SS1 phenotype in THP-1 cells was previously described (6, 9). Briefly, LPS tolerance is generated by an initial stimulation with Escherichia coli O111:B4 LPS (1.0 μg/ml) for 16 h, followed by restimulation (1.0 μg/ml) for the times shown in Fig. 1 to 3. This LPS acts exclusively through TLR4 as determined with cells lacking TLR4. High concentrations of LPS are used to optimize the tolerance phenotype, although changes occur with doses as low as 10 to 100 ng/ml. Tolerance occurs within 3 h and is sustained for at least 96 h (unpublished data). LPS-induced tolerance is typified by repression of acute proinflammatory genes and sustained expression of anti-inflammatory and antimicrobial genes. Normal and LPS-tolerant THP-1 cells (1.0 × 10⁵ cells/sample) were washed once with RPMI 1640 medium, resuspended in fetal bovine serum-supplemented RPMI 1640 medium at 1 × 10⁶ cells/ml, and stimulated with LPS (1.0 μg/ml) for the times shown in Fig. 1 to 3. Low-passage-number and log-phase cells were used for all experiments.

**Peripheral blood leukocyte (PBL) isolation from SS1 patients and healthy participants.** The selection of patients with SS1 was based on the modified physiologic criteria of Bone (3) as previously described (18). Based on these criteria, 95% of patients with sepsis were reproducibly shown to have reduced IL-1β and TNFα production in response to LPS (19). The majority of patients enrolled in the present study were found positive for gram-positive and/or gram-negative bacteria. SS1 in humans is not limited, however, to infection and may be associated with systemic inflammatory response syndrome in critically ill patients. Therefore, the study for clinical research associated with the general clinical research center of the medical center.

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RESULTS

LPS induces sustained transcription of IκBα during LPS-mediated tolerance. Our previous report indicated that IκBα protein levels are sustained in LPS-tolerant THP-1 cells with repressed transcription of IL-1β, suggesting persistent transcription of IκBα in contrast to repressed transcription of acute proinflammatory genes (15). To test whether IκBα transcription persists following LPS-induced tolerance, we measured IκBα mRNA levels in LPS-naive and LPS-tolerant cells, as shown in Fig. 1A. As assessed by real-time PCR, IκBα mRNA levels in normal cells stimulated with LPS rapidly and dramatically increased eightfold, diminished somewhat, and then remained substantially elevated over the 12-h test. IκBα mRNA levels remained elevated at the 12-h time point (e.g., the 0-h time point of tolerant cells is shown in Fig. 1A). Following the second LPS stimulation, IκBα mRNA slightly but significantly increased in tolerant THP-1 cells, after which levels remained elevated. To ensure that transcription was the major mechanism controlling the steady-state level of IκBα mRNA, we also measured the IκBα mRNA half-lives in normal and tolerant cells and found no significant differences (Fig. 1B). Together, the data support sustained transcription of IκBα RNA during LPS-mediated gene tolerance.

We previously reported that IκBα protein, which is prominent in unstimulated normal and LPS-tolerant THP-1 cells, is rapidly degraded and resynthesized following LPS stimulation of both LPS-responsive and LPS-tolerant cells (29). We confirmed this paradigm in this study, as shown in Fig. 1C. Protein production persisted in unstimulated normal and tolerant cells (Fig. 1C, lane 2). Using actinomycin D to block de novo IκBα synthesis and to follow decay, we confirmed the high turnover rate of IκBα in unstimulated normal and tolerant THP-1 cells (data not shown). When normal cells were stimulated by LPS, IκBα protein levels diminished quickly and then increased. In tolerant THP-1 cells, the IκBα level following LPS stimulation appeared to diminish somewhat less than that observed in normal cells and increased thereafter (Fig. 1C). The steady-state levels did not closely parallel the increases in IκBα mRNA noted after LPS stimulation (Fig. 1A and C, lane 4), supporting the constantly high turnover rate of the protein in the basal free state (21). Together, the IκBα mRNA and protein data support sustained expression of the IκBα gene during LPS-mediated gene reprogramming.

Differential binding of NF-κB p65 and RelB transcription factors to the IκBα core promoter occurs in normal and LPS-tolerant cells. A previous report showed that p65, p50, and
RelB may differentially alter LPS-induced transcription in dendritic cells through a process of dimer exchange, wherein the rapidly formed p65/p50 dimer is replaced by slowly formed RelB/p50 or RelB/p52 (22). To test whether such a process might occur in the LPS-tolerant THP-1 cell model of SSI, we used a ChIP assay to assess the binding of p65, p50, p52, and RelB to the NF-κB site at the core promoter, the functionally important NF-κB site for the activation of IκBα transcription (13). We observed no detectable nonspecific binding of matched immunoglobulin G to the IκBα promoter (not shown). Real-time PCR and standard PCR analyses of ChIP DNA revealed that NF-κB p65 was enhanced at the IκBα promoter following LPS stimulation in LPS-responsive cells. The binding was increased threefold by 60 min and then diminished slightly over 3 h (Fig. 2A). The elevated NF-κB p65 binding correlated with the increased IκBα mRNA transcription with LPS stimulation in normal cells, as shown in Fig. 1A. p65 binding was also detected in LPS-tolerant cells prior to stimulation with a second dose of LPS. In contrast to control cells, LPS stimulation of tolerant cells resulted in a significant reduction in p65 binding to the IκBα promoter (Fig. 2A). NF-κB p50 binding to the IκBα promoter remained constant in unstimulated and LPS-stimulated normal cells. LPS stimulation of LPS-tolerant cells resulted in an increase in p50 binding by 3 h (Fig. 2B). Limited RelB binding to the IκBα core promoter was detected in normal cells for up to 3 h,

![Graphs showing binding of p65, p50, and RelB to the IκBα promoter in normal and LPS-tolerant cells.](image-url)
without or with LPS stimulation (Fig. 2C). This was expected, since RelB is minimally expressed in LPS-naive THP-1 cells until 6 h (31). RelB promoter binding was increased fourfold in LPS-tolerant cells 16 h after the initial LPS dose and prior to the secondary LPS stimulation. LPS secondary stimulation of tolerant cells induced rapid and transient increases in RelB promoter binding at 30 min and 1 h after LPS stimulation, which occurred coincident with reduced p65 binding. RelB promoter binding then returned to a level similar to that in unstimulated LPS-tolerant cells (Fig. 2C). We did not detect binding of p52 to the IκBα promoter (not shown). Together, these data supported an exchange of the p65/p50 dimer with RelB/p50.

IκBα transcription in tolerant THP-1 cells requires RelB. Our ChIP and mRNA data suggested that RelB might function as a transcription activator at the IκBα gene promoter. To test this, we used RelB siRNA to deplete RelB in LPS-tolerant cells. Western blot analysis confirmed that RelB siRNA knockdown virtually eliminated RelB protein in whole-cell, cytosol, and nuclear extracts. LDH, lactate dehydrogenase; sumo, small ubiquitin-related modifier protein. (A) LPS induction of IκBα transcription was markedly reduced in RelB siRNA-transfected cells compared with control siRNA. Results using normal, untransfected cells stimulated with LPS, where there was the expected rapid induction of IκBα transcription, are compared. Real-time PCR results are presented as the increase in mRNA copies relative to that of normal cells at 0 h (set as an arbitrary unit of one), as analyzed by quantitative real-time PCR. (C) ChIP assays confirmed that RelB binding to the IκBα promoter was diminished concomitantly with a reduction in IκBα transcription following RelB siRNA knockdown and loss of RelB protein. Data are the means ± standard errors of the means of three independent experiments. *, significant difference ($P < 0.05$).
down of RelB protein in tolerant cells resulted in a reduction in RelB binding to the \( \text{I} \beta \text{B} \) proximal promoter (Fig. 3C), further supporting RelB as a transcription activator in tolerant cells.

**RelB binding at the \( \text{I} \beta \text{B} \) core promoter is increased in PBLs of SSI patients.** Our report of studies of PBLs obtained from patients with SSI shows that RelB protein expression is markedly increased but that little or no RelB is detectable in normal PBLs (31). To test whether RelB might participate in sustaining activation of \( \text{I} \beta \text{B} \) in human SSI, in support of the concept for the paradigm observed for LPS-mediated tolerance of THP-1 cells, we used ChIP to assess in vivo RelB binding to the \( \text{I} \beta \text{B} \) promoter in normal PBLs and in PBLs from patients with SSI. We found prominent RelB binding to the \( \text{I} \beta \text{B} \) promoter in PBLs obtained from three patients with SSI (Fig. 4).

**DISCUSSION**

Our general conclusion from this study is that de novo induction of RelB is required to sustain transcription of \( \text{I} \beta \text{B} \) following LPS-induced tolerance of gene expression in THP-1 promonocytic cells, a model of changes that occur in circulating leukocytes during SSI. This interpretation is supported by several findings. First, persistent transcription and translation of \( \text{I} \beta \text{B} \) mRNA occurred following LPS tolerance, and \( \text{I} \beta \text{B} \) mRNA further increased after secondary LPS stimulation of cells in which RelB had been induced. Although changes in \( \text{I} \beta \text{B} \) protein levels did not precisely parallel levels of mRNA, the rapid repletion of the protein following its LPS-induced proteasome degradation in normal or tolerant cells supports the fact that a persistent state of active transcription existed. The observation that levels of \( \text{I} \beta \text{B} \) protein did not precisely follow levels of \( \text{I} \beta \text{B} \) mRNA was not surprising, since we have reported that transcriptional and translational events may be under distinct controls in LPS-tolerant cells (16).

A second level of support for our concept relates to the changes that occur in the NF-κB binding to the \( \text{I} \beta \text{B} \) promoter. LPS stimulation of normal cells resulted in rapid formation of the transcriptionally active p65/p50 dimer at the \( \text{I} \beta \text{B} \) core promoter, which supported an initial wave of increased transcription. During this time, there was little RelB available. After induction of RelB by the initial LPS dose which made the cells tolerant, there was an accumulation of RelB at the \( \text{I} \beta \text{B} \) proximal promoter, during which time there was sustained transcription. When LPS-tolerant cells were stimulated with LPS a second time, there was rapid reduction of p65 binding (i.e., p65/p50 dimer formation), during which time RelB binding to the \( \text{I} \beta \text{B} \) promoter further increased. This was associated with an apparent RelB/p50 dimer exchange for p65/p50.

A third level of support for our general conclusion was the fact that significant reductions in \( \text{I} \beta \text{B} \) mRNA levels occurred in LPS-tolerant cells following depletion of RelB protein through the siRNA process. The low levels of mRNA observed in LPS-tolerant cells after siRNA knockdown were paralleled by reductions in \( \text{I} \beta \text{B} \) protein (not shown) and with a concomitant reduction in RelB binding to the \( \text{I} \beta \text{B} \) proximal promoter. These data support a loss of the transactive RelB/p50 dimer when RelB is depleted. Finally, the concept that RelB may function as a transcription activator in human SSI is supported by our finding that RelB also bound to the \( \text{I} \beta \text{B} \) proximal promoter in circulating leukocytes obtained from three patients with SSI. RelB binding was not detected on the \( \text{I} \beta \text{B} \) promoter of control blood leukocytes.

Taken together with our previous report of RelB-dependent repression of IL-1β and TNF-α (9, 31), our data support the observation that RelB regulated distinct sets of genes in the LPS-tolerant THP-1 model of SSI. Unlike transcriptionally repressed acute proinflammatory IL-1β and TNF-α promoters that bind heterochromatin modifier HP1α in tolerant THP-1 cells (9), no binding of HP1α to the \( \text{I} \beta \text{B} \) promoter was found in this study (data not shown). This supports the finding that \( \text{I} \beta \text{B} \) persists as euchromatin during gene tolerance, at a time when IL-1β and TNF-α are transformed to silenced heterochromatin. Further support for the euchromatic nature of the \( \text{I} \beta \text{B} \) gene in LPS-tolerant cells is our unpublished observation that the histone code mark for active transcription, histone H3 phosphorylation on serine 10, increases following LPS stimulation of tolerant THP-1 cells.

During LPS activation of macrophages, distinct NF-κB factors, such as p65, p50, c-Rel, p52, and RelB, may dynamically interact with the promoter and enhancers of the same gene (23). These transcription factors may bind concomitantly or replace each other by dimer exchange (4). Dimer exchanges between RelB, p65, and promoter-bound p50 occur in dendritic cells following LPS stimulation by a process that can both repress transcription of IL-12p40 and activate transcription of IL-8 (22). Our study supports a similar paradigm contributing to LPS tolerance in the SSI phenotype.

LPS stimulation of normal cells, LPS-tolerant cells, or SSI circulating leukocytes leads to \( \text{I} \beta \text{B} \) rapid cytoplasmic degradation with concomitant release and accumulation of p65 and p50 within the nucleus (14). However, the accumulating nuclear p65 cannot bind to the promoters of repressed acute proinflammatory genes in LPS-tolerant cells due to the heterochromatin-based silencing mechanisms (6, 9, 31). Still, the nuclear p65 would be available to rapidly bind to genes that are not repressed and remain as transcriptionally responsive as euchromatin, such as \( \text{I} \beta \text{B} \). The delayed RelB induction by LPS would later support the sustained expression of the \( \text{I} \beta \text{B} \) gene through dimer exchange, even when p65 is removed from the promoter and resequenced with the cytoplasmic \( \text{I} \beta \text{B} \) (20). Accordingly, the dimer exchange to a RelB/p50 activator in
could sustain expression of the IκBα gene, since the RelB/p50 dimers cannot be sequestered and rendered inactive by IκBα (22). The occurrence of NF-κB dimer exchange supports the observation that preexisting promoter complexes are disassembling and being reassembled based on available new components. This renders genes sensitive to the expression and concentrations of other NF-κB proteins and cooperating factors.

The mechanisms responsible for the dual RelB functions of activation and repression and its coupling to epigenetic modifications during LPS tolerance are unknown but are of considerable importance. RelB likely mediates different epigenetic modifications at distinct sets of promoters. Promoter specificity could reside in the different DNA structures, the interaction of RelB with distinct coactivators or repressors (8, 12), the necessity of optimal RelB/p50 DNA-binding motifs. Biochem. Biophys. Res. Commun. 365:583–588.

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