Epigenetic Silencing of Tumor Necrosis Factor \( \alpha \) during Endotoxin Tolerance*

Mohamed El Gazzar\textsuperscript{1,1}, Barbara K. Yoya\textsuperscript{1,5}, Jean Y.-Q. Hu\textsuperscript{1}, Sue L. Cousart\textsuperscript{1}, and Charles E. McCall\textsuperscript{1*}

From the \textsuperscript{1}Department of Internal Medicine, Section of Molecular Medicine, and \textsuperscript{1}Department of General Surgery, Wake Forest University School of Medicine, Winston-Salem, North Carolina 27157

Sustained silencing of potentially autotoxic acute proinflammatory genes like tumor necrosis factor \( \alpha \) (TNF\( \alpha \)) occurs in circulating leukocytes following the early phase of severe systemic inflammation. Aspects of this gene reprogramming suggest the involvement of epigenetic processes. We used THP-1 human promonocytes, which mimic gene silencing when rendered endotoxin-tolerant \textit{in vitro}, to test whether TNF\( \alpha \) proximal promoter nucleosomes and transcription factors adapt to an activation-specific profile by developing characteristic chromatin-based silencing marks. We found increased TNF\( \alpha \) mRNA levels in endotoxin-responsive cells that was preceded by dissociation of heterochromatin-binding protein 1alpha, demethylation of nucleosomal histone H3 lysine 9 (H3(Lys9)), increased phosphorylation of the adjacent serine 10 (H3(Ser10)), and recruitment of NF-\( \kappa \)B RelA/p65 to the TNF\( \alpha \) promoter. In contrast, endotoxin-tolerant cells repressed production of TNF\( \alpha \) mRNA, retained binding of heterochromatin-binding protein 1alpha, sustained methylation of H3(Lys9), reduced phosphorylation of H3(Ser10), and showed diminished binding of NF-\( \kappa \)B RelA/p65 to the TNF\( \alpha \) promoter. Similar levels of NF-\( \kappa \)B p50 occurred at the TNF\( \alpha \) promoter in the basal state, during active transcription, and in the silenced phenotype. RelB, which acts as a repressor of TNF\( \alpha \) transcription, remained bound to the promoter during silencing. These results support an immunodeficiency paradigm where epigenetic changes at the promoter of acute proinflammatory genes mediate their repression during the late phase of severe systemic inflammation.

Gene reprogramming during severe systemic inflammation generates, among other patterns, silencing of acute proinflammatory genes, such as TNF\( \alpha \) and IL-1\( \beta \), that initiate acute systemic inflammation and damage to multiple organs (1, 2). The silencing of acute proinflammatory genes, which normally follows an initial activation phase (3), is clinically relevant in humans because it participates in generating an acquired state of immunodeficiency that correlates with poor prognosis and increased mortality (4). Gene silencing as a result of disrupted transcription occurs in circulating and tissue leukocytes during severe systemic inflammation in animals and humans (2, 5, 6). The silenced component of gene reprogramming is characterized by a tolerance to endotoxin and can persist for days or even weeks (5). Endotoxin tolerance is defined by the repressed expression of a set of proinflammatory genes in response to the stimulation of the Toll-like receptor 4 by endotoxin. Endotoxin tolerance is constitutively present in blood leukocytes obtained from humans and animals with severe systemic inflammation and can be generated \textit{in vitro} by using endotoxin as a primary stimulus of macrophages (7).

The complex mechanisms responsible for gene silencing are regulated at many levels and continue to emerge. At the level of chromatin, covalent modifications of the NH\( _2 \)-terminal tails of the four core histones (H2A, H2B, H3, and H4) play an essential role in both activating or silencing gene expression (8, 9). These modifications account for a “histone code” that generates specific binding sites for proteins that regulate both chromatin structure and transcription (10). Well characterized histone modifications associated with transcriptional regulation and activation include H3 phosphorylation of Ser\textsuperscript{10}, demethylation of Lys\textsuperscript{9}, and acetylation of Lys\textsuperscript{9} and Lys\textsuperscript{14} through the concerted activity of some known and many as yet unidentified mediators (11, 12). For example, acetylation of histone on lysine residues relaxes the compact structure of the nucleosome and activates transcription, whereas histone deacetylation represses transcription (13). In addition, methylation of histone H3 on Lys\textsuperscript{9} facilitates DNA methylation, heterochromatin formation, and gene silencing (14), whereas phosphorylation of histone H3 on Ser\textsuperscript{10} correlates with activation of immediate response genes of which many acute proinflammatory proteins can be included (13, 15–17). This supports a chromatin-based “phosphorylation/methylation switch” where phosphorylation of histone residue adjacent to a methylation mark parallels a loss of binding of negative factors accompanied by the demethylation of that residue and the subsequent recruitment of transcription factors and co-activating proteins and gene activation (18). This binary switch may also play a negative role through recruitment of co-repressors such as the heterochromatin protein HP1, which also binds to methylated lysines on H3 and contributes to gene silencing by establishing heterochromatin formation (19). These changes in chromatin structure contain a molecular imprint...
that may underlie cell memory and epigenetic inheritance of cell progeny.

Chromatin remodeling influences the activity of transcription factors, such as members of the NF-κB family (20). Post-translational modifications influence the transcription potential of NF-κB (21–23) and can be critical for chromatin-associate activity and DNA binding of NF-κB p65 as a heterodimer with p50 and for its assembly with IκBα and nuclear export (24, 25). For example, phosphorylation of NF-κB p65 at Ser276 and histone H3 at Ser10 by MSK1 has been associated with activation of IL-6 promoter (26, 27). Many if not all acute proinflammatory genes are regulated, at least in part, by NF-κB, and we reported changes in NF-κB binding that are associated with the silencing of IL-1β expression during endotoxin tolerance (28).

TNFα plays a pivotal role in coordinating host defense against infection (29) and is a crucial contributor to the pathophysiology of severe systemic inflammation and septicemia (30). Regulation of human TNFα in monocytes is complex and involves transcriptional and post-transcriptional mechanisms (31). Its expression is silenced in blood leukocytes during severe systemic inflammation (1). The silencing can be mimicked in normal monocytes or THP-1 monocytes by inducing endotoxin tolerance in vitro (2, 5, 33).

We investigated the mechanism of TNFα gene silencing by comparing endotoxin-responsive with endotoxin-tolerant THP-1 cells. We report that epigenetic changes and formation of heterochromatin marks occurred during TNFα silencing. These include sustained binding of HP1α and methylation of H3(Lys9), reduced phosphorylation of H3(Ser10), and disrupted recruitment of NF-κB RelA/p65 to the TNFα promoter. RelB also bound to the TNFα promoter and acted as a transcription repressor during gene silencing.

MATERIALS AND METHODS

Cell Culture and Induction of LPS Tolerance—The human promonocytic cells, THP-1, obtained from the American Type Culture Collection were maintained in RPMI 1640 medium (Invitrogen) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 10% fetal bovine serum (HyClone, Logan, UT) in a humidified incubator with 5% CO2 at 37°C. For induction of tolerance (7), cells were incubated for 16 h with 1 μg/ml Gram-negative bacterial LPS (Escherichia coli 0111:B4; Sigma). LPS-tolerant and LPS-responsive (normal) cells were washed with minimal medium, cultured at 1 × 106 cells/ml, and stimulated with 1 μg/ml LPS for the indicated times.

TNFα Enzyme-linked Immunosorbent Assay—The levels of total (intracellular and secreted) TNFα proteins were measured by enzyme-linked immunosorbent assay according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN). The samples were assayed in duplicate. The minimal detection limit of the assay was 1.6 pg/ml.

Chromatin Immunoprecipitation (ChIP) Assay—ChIP assay was performed to assess NF-κB and modified histone protein binding to the TNFα promoter using the ChIP assay kit according to the manufacturer’s instructions (Active Motif, Carlsbad, CA). Briefly after stimulation, cells were fixed in 1% formaldehyde (to cross-link DNA-proteins) for 10 min at room temperature. Cross-linked chromatin was isolated and sheared with an enzyme mixture for 15 min at room temperature. These conditions generate chromatin fragments containing DNA ranging in size from 200 to 500 bp. The sheared chromatin was recovered by centrifugation at 12,000 rpm at 4°C. The chromatin solution was pre-cleared with salmon sperm DNA/protein G-agarose for 2 h at 4°C. Ten microliters of the chromatin solution were reserved as “input” sample. The remaining chromatin was immunoprecipitated by incubation at 4°C overnight with antibodies specific to NF-κB p65, p50, RelB (Santa Cruz Biotechnology, Santa Cruz, CA), HP1α, total histone H3, phosphohistone H3 (p-H3(Ser10)), or dimethylhistone H3 (me2-H3(Lys4)) (Upstate Biotechnology, Lake Placid, NY). The immunoprecipitated complexes were further incubated for 1 h at 4°C with protein G-agarose, washed, and then eluted in a buffer containing 1% SDS and 0.1 M NaHCO3. The immunoprecipitated and input sample cross-links were reversed by heating at 65°C overnight. The resulting DNA was washed in a column and recovered in 100 μl of H2O.

Semiquantitative PCR—PCR was performed in a 50-μl volume containing 5 μl of ChIP DNA, a 1 μM concentration of each primer, 2 mM MgCl₂, 0.2 μM dNTPs, and 0.04 units/μl AmpliTaq Gold DNA polymerase (Applied Biosystems). The PCR conditions were as follows: 1 cycle at 94°C for 5 min; 35 cycles at 94, 58, and 72°C for 30 s each; and a final cycle at 72°C for 5 min. Equal amounts of PCR products were run on a 1.2% ethidium bromide-stained agarose gel, and images were captured using a Quantity One imager (Bio-Rad). The primers used in PCR were designed to amplify a sequence in the human TNFα proximal promoter region containing the κB3 site at −98 bp relative to the transcription start site (34) and were as follows: TNFα κB3 forward (5′-TACGCTCTCCCTCACATGGAG-3′) and reverse (5′-TGCTGGCTGGTGTTGCCAAA-3′).

Quantitative Real Time PCR—Real-time PCR was performed to precisely quantify the TNFα promoter sequence in the ChIP DNA as well as the TNFα mRNA expression.

For ChIP DNA analysis, the same primers described above were used with an internal fluorogenic probe that was labeled with the fluorescent dyes carboxyfluorescein (FAM) as a reporter dye and N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA) as a quencher dye (5′-FAM-CTTGGTG-GAGAAAACC-TAMRA-3′) (Applied Biosystems). The PCR (25 μl) contained 5 μl of ChIP DNA, 12.5 μl of 2× TaqMan Universal Master Mix containing DNA polymerase and dNTPs, a 300 nM concentration of each primer, and 100 nM internal probe. The PCR conditions were 2 min at 50°C and 10 min at 95°C followed by 40 cycles with 15 s at 95°C and 1 min at 60°C (combined annealing and extension) using an ABI Prism 7000 Sequence Detection System (Applied Biosystems). An isotype-matched IgG-immunoprecipitated DNA sample was also amplified as a negative control (data not shown). Sample data were normalized to the input DNA and are presented as -fold change relative to DNA from untreated cells (0 h).

For TNFα mRNA analysis, total RNA was isolated using RNA STAT-60 according to the manufacturer’s protocol (Tel-Test, Friendswood, TX). Two micrograms of RNA were reverse transcribed to cDNA in a 25-μl volume containing 0.2
μM dNTPs, 2.5 μM oligo(dT), 5 mM MgCl₂, and 0.25 units/μl murine leukemia reverse transcriptase (Applied Biosystems). The PCR was performed using 5 μl of cDNA and TNFα and glyceraldehyde-3-phosphate dehydrogenase pre-designed TaqMan primer/probe kits (Applied Biosystems) under the conditions described above. Sample data were normalized to glyceraldehyde-3-phosphate dehydrogenase mRNA and are presented as -fold change relative to mRNA from untreated cells.

RNA Interference—Control and RelB-specific small interfering RNAs (siRNAs; Santa Cruz Biotechnology) were transfected by electroporation with 5 μl (0.5 μM) of siRNA in 100 μl of transfection medium (Nucleofector; Amaxa, Gaithersburg, MD). Immediately after transfection, cells were left unstimulated or were stimulated with 1 μg/ml LPS to induce tolerance. After 48 h, cells were harvested, washed with minimal medium, and stimulated for 3 h with 1 μg/ml LPS. Cells were harvested, and RNA was isolated and used to determine the TNFα mRNA expression levels.

Statistical Analysis—Data were analyzed by Microsoft Excel 2003 and are presented as the mean ± S.E. from at least three independent experiments. Student’s t test was used to determine significant differences between groups. p values <0.05 were considered statistically significant.

RESULTS

TNFα Protein and mRNA Expression Are Silenced in LPS-tolerant THP-1 Cells—The experimental model used in this study mimics the LPS-tolerant phenotype that occurs in blood leukocytes during the course of severe systemic inflammation in animals and sepsis in humans (35, 36) where cytokine expression, including that of TNFα, is silenced after the initial induction phase following LPS exposure (4, 6, 37). We first measured the TNFα protein and mRNA levels in LPS-responsive and LPS-tolerant cells. As shown in Fig. 1, the total TNFα protein level was significantly increased by 3 h poststimulation and remained elevated at 8 h. With the longer incubation times (data not shown), we observed a gradual decline in the protein levels starting at 10 h. In contrast, LPS-tolerant cells produced TNFα protein at a background level throughout the course of stimulation.

We also determined TNFα mRNA expression levels by real time PCR as shown in Fig. 1. TNFα mRNA was rapidly and significantly induced in responsive cells after 0.5 h of stimulation with LPS. The mRNA levels were dramatically decreased at 3 h. Consistent with the low protein levels, we detected very low TNFα mRNA levels in LPS-tolerant cells. Together these data demonstrate that TNFα protein and mRNA are induced by LPS in responsive THP-1 cells but are silenced in LPS-tolerant THP-1 cells.

Histone H3 Phosphorylation on Ser10 and Dimethylation on Lys9 of the TNFα Promoter Are Induced by LPS in Responsive but Not LPS-tolerant Cells—To investigate whether histone modifications contribute to TNFα silencing during LPS tolerance, we examined histone H3(Ser10) phosphorylation and H3(Lys9) dimethylation levels at the TNFα promoter using ChIP DNA from responsive and tolerant THP-1 cells. We found that in untreated LPS-responsive cells, histone H3 was phosphorylated on Ser10 at low levels. Ser10 phosphorylation was significantly increased at 1 and 4 h after LPS stimulation and returned to a near basal level by 4 h (Fig. 2, A and B). In LPS-tolerant cells, histone H3(Ser10) phosphorylation was only slightly increased at 1 h. We also found that histone H3 dimethylation on Lys9 was significantly decreased in LPS-responsive cells by 0.5 h after LPS stimulation and gradually returned to its basal state at 4 h (Fig. 2, A and B). In LPS-tolerant cells, histone H3(Lys9) was constitutively dimethylated and remained almost unchanged following LPS stimulation (Fig. 2, A and C). These data show a phosphorylation/methylation binary switch activity in responsive THP-1 cells that is impaired in silenced, LPS-tolerant THP-1 cells.

HP1α Dissociates from the TNFα Promoter in LPS-responsive but Not LPS-tolerant Cells—The data presented above demonstrated that H3(Lys9) was persistently methylated in tolerant cells and that this histone modification correlated with TNFα silencing. Proteins that specifically interact with post-translational modifications of histone play a crucial role in mediating the functional role of histone marks (38, 39). Methylation of the Lys9 of histone H3 is an epigenetic mark for formation of heterochromatin and associated gene silencing (19, 40). This phenotype is often associated with binding of repressor HP1α or -β.
wherein the chromodomain binds to methylated H3(Lys9) and participates in gene silencing by establishing heterochromatin (19, 40). We investigated whether HP1α/H9251 is recruited to TNFα/H9251 promoter during endotoxin silencing. Standard and real time PCR analysis of ChIP DNA using HP1α/H9251 antibody showed that HP1α/H9251 bound at the basal level in unstimulated cells (Fig. 3). The amount of HP1α/H9251 bound to the promoter was significantly decreased in responsive cells after 0.5 h of LPS stimulation before it returned to a near basal level by 4 h. In contrast, HP1α remained bound in tolerant cells consistent with a role in repression of TNFα and contributing to the silenced phenotype of LPS tolerance.

**FIGURE 2.** LPS-tolerant cells exhibit a different histone modification pattern. ChIP assay was performed to monitor histone H3 phosphorylation and methylation at the TNFα promoter. Cross-linked chromatin was isolated and immunoprecipitated with antibodies specific to total histone H3, phosphorylated histone H3 (p-H3(Ser10)), and dimethylated histone H3 (me2-H3(Lys9)). Samples isolated before immunoprecipitation (Input) were used as internal controls. The presence of TNFα promoter sequences in the ChIP DNA (which reflects the relative in vivo binding) was analyzed by standard PCR (A) and real time PCR (B and C) using primers that flank the NF-κB (κB3) binding site at −98 bp followed by ethidium bromide staining. Data were normalized to total H3 and are presented as fold change relative to DNA from unstimulated cells (0 h). Results in B and C are mean ± S.E. from at least three independent experiments. *, p < 0.05.

**FIGURE 3.** HP1α persistently binds the chromatin during endotoxin tolerance. Results of standard (A) and real time (B) PCR showing HP1α binding to TNFα promoter are shown. ChIP assay and PCR were performed as described under “Materials and Methods.” Data were normalized to input DNA and are presented as fold change relative to DNA from unstimulated cells (0 h). Results in B are mean ± S.E. from at least three independent experiments. *, p < 0.05.

**TABLE**

| Time in LPS (h) | Responsive | Tolerant |
|-----------------|------------|----------|
| 0               | 0.5        | 1        | 4        |
| Input           |            |          |          |
| total H3        |            |          |          |
| p-H3(Ser10)     |            |          |          |
| me2-H3(Lys9)    |            |          |          |

**FIGURE 4.** LPS-induced NF-κB p65 Binding to the TNFα Promoter Is Inhibited in LPS-tolerant Cells—Human TNFα/H9251 promoter has three NF-κB binding sites, κB1, κB2, and κB3, known to be involved in its transcription regulation (34, 41, 42). Promoter proximal transcription binding sites are usually functionally associated with chromatin-based gene silencing. We examined the kinetics of NF-κB protein binding to the NF-κB binding site κB3 in the TNFα promoter. We focused our investigation on the κB3 site because of its close proximity to the transcription initiation site and the assembly of basal transcription machinery. Also this site binds NF-κB proteins and contributes to the induction of TNFα in THP-1 cells (30, 43). Standard PCR and quantitative real time PCR analysis of ChIP DNA revealed a basal level binding by p65 in unstimulated cells (Fig. 4). Upon LPS stimulation, we detected a significant increase (~11-fold) in p65 binding at 0.5 and 1 h following LPS stimulation (Fig. 4, A and B) that returned to near basal levels by 4 h. In LPS-tolerant cells, however, p65 binding occurred at the basal level and was not notably induced by LPS (Fig. 4, A and B). The low level binding of p65 at the TNFα promoter in tolerant cells occurs despite normal expression, nuclear translocation, and increased levels of p65 in LPS-tolerant THP-1 cells (44). We detected low levels of p50 binding to the TNFα promoter in both responsive and tolerant cells that were not significantly changed in response to LPS stimulation (Fig. 4, A and C).
RelB Binds to the TNFα Promoter in LPS-tolerant Cells, and RelB Knockdown Reverses Tolerance and Restores TNFα mRNA Induction by LPS—We previously reported that RelB, which is not expressed in the basal state in THP-1 cells, is induced following LPS stimulation and subsequently acts as a transcription repressor (44). A similar paradigm may occur in human blood leukocytes during severe systemic inflammation (44). To examine whether RelB, another NF-κB family member, participates in silencing of TNFα in LPS-tolerant cells, we first analyzed its in vivo binding to the TNFα promoter. We found that RelB binds to TNFα promoter in responsive cells. Such binding slightly increased by 4 h of LPS stimulation (Fig. 4, A and D). In contrast, a significant increase in RelB binding occurred in LPS-tolerant cells and further increased by 4 h of LPS stimulation (Fig. 4, A and D).

Using RelB-specific siRNA, we further tested whether decreasing RelB could restore TNFα expression in tolerant cells. As shown in Fig. 5, transfection of RelB siRNA reversed LPS tolerance and restored the LPS-induced TNFα mRNA expression to a level close to that seen in LPS-responsive cells (compare responsive + LPS with tolerant + LPS).

In summary, we found that TNFα gene expression induced by LPS is associated with the activation of the phosphorylation/methylation binary switch wherein histone H3 becomes phosphorylated on Ser10 and demethylated on the adjacent Lys9. In contrast, a cell model of severe systemic inflammation and pheno type-specific proinflammatory gene silencing is associated with a deficiency in the binary switch and a persistent methylation in the TNFα promoter. This epigenetic silencing is characteristic of the formation of heterochromatin and included persistently bound HP1α. Changes in NF-κB binding to chromatin associated with silenced TNFα promoter were also observed, specifically decreased binding of NF-κB p65 and increased binding of the NF-κB repressor RelB.

DISCUSSION

Chromatin configuration imposes profound and lasting effects on transcription (8). Changes in chromatin nucleosome structure due to histone modifications regulate transcription of many genes by facilitating the interactions of transcription-activating or -repressing factors and the general transcriptional machinery. This mechanism operates for inflammatory genes such as IL-10, IL-4, IL-12p40, IL-5, TNFα, and MCP-1 (45–50).

Dysregulated transcription, a salient feature of the endotoxin-tolerant phenotype associated with severe sepsis, results in reprogramming of gene expression with sustained silencing of LPS-induced proinflammatory gene expression, which follows a burst in expression of autotoxic cytokines like TNFα (1). In this study, we found that the dysregulated transcription activation of TNFα expression during LPS tolerance and gene silencing is associated with chromatin modifications characterized by epigenetic silencing “marks” that are typically associated with formation of heterochromatin (15, 18, 51, 52). The evidence for this epigenetic imprint includes persistent dimethylation of H3(Lys9) and recruitment of HP1α to the proximal promoter coupled with reduced phosphorylation of H3(Ser10). There was concomitant disruption of recruitment of NF-κB p50 to the proximal promoter, but NF-κB p50 remained bound, possibly as a homodimer. The silenced phenotype was also associated with recruitment of RelB to the proximal promoter where RelB facilitates transcription repression. In contrast, LPS-responsive cells showed the active transcriptional marks of dimethylation of H3(Lys9), phosphorylation of H3(Ser10), and binding of NF-κB p65 in the presence of p50, which likely represents a heterodimer.

Phosphorylation of H3 on Ser10 is usually a transcription activation mark, which parallels the displacement of the HP1α and -β repressor proteins and their interactions with auxiliary factors such as histone demethylases, which can demethylate H3(Lys9) (53–56). This nucleosome-based process may enhance accessibility of NF-κB sites (26) and increase recruitment of general transcription machinery (57). The nucleosomal “shift” and assembly of transcription factors may be independent. There are reports of connections between transcription activation and phosphorylation at H3(Ser10) and recruitment of NF-κB p65 to proximal promoter sites (58, 59). If so,
TNFα Silencing during Endotoxin Tolerance

FIGURE 5. RelB gene silencing by siRNA restores TNFα mRNA expression in LPS-tolerant THP-1 cells. Cells were transfected with control (nonspecific) or RelB-specific siRNA and then left unstimulated or stimulated with 1 μg/ml LPS for 48 h. Responsive (r) and LPS-tolerant (t) cells were washed and left unstimulated or stimulated with 1 μg/ml LPS for 3 h. TNFα mRNA expression was analyzed by real time reverse transcription-PCR. The results are presented as -fold change in TNFα mRNA relative to LPS-responsive (r−) cells (set as 100% arbitrary unit). Data are the mean ± S.E. from at least three independent experiments. *p < 0.05, t− versus t+. Notice the marked increase in TNFα mRNA after RelB siRNA transfection (compare t+ with r+).

the precise mechanisms for interdependency are unknown, but several studies suggest that IκB Kinase α may be central in this pathway by phosphorylating both histone H3(Ser10) and NF-κB p65 (58–60).

H3(Lys3) di- or trimethylation, on the other hand, has been linked to the assembly of heterochromatin through HP1α or -β binding to methylation sites to sustain gene repression, which may be passed during mitosis (51). Silencing cofactors such as histone methyltransferases and DNA CpG methylases as well as other recruited co-repressors may cooperate in this process (61–63). For example, artificial targeting of HP1 to euchromatin (transcriptionally active) sites significantly induces gene repression and local chromatin condensation coincident with recruitment of other chromatin-modifying proteins (64, 65).

The disrupted binding of NF-κB p65 to the proximal promoter we observed during silencing likely occurs at the κB3 binding sequence. This sequence near the initiation site is active in transcription of TNFα in THP-1 cells as assessed by transfection analysis (30, 43). We cannot exclude that more distal sites on enhancer regions of TNFα (30, 42, 43, 66, 67) are also involved in gene activation and silencing, but transcription-related DNA sequences near the initiation sites of the promoter are connected to the assembly of the general transcription machinery. In any event, disrupted binding of NF-κB p65 provides an explanation for reduced transcription of acute proinflammatory genes during severe endotoxin tolerance and severe systemic inflammation, whereas nuclear levels of NF-κB p65 are increased (5, 37, 68). Our results also showed sustained NF-κB p50 binding to TNFα promoter in both responsive and LPS-tolerant cells. This supports that p50 binding to the TNFα promoter in tolerant cells may support repression by forming p50-p50 homodimers (29, 42, 43).

Our observations that RelB acts as a repressor during endotoxin tolerance extends our similar findings at the IL-1β promoter during silencing (44). It is not yet clear how RelB represses transcription. RelB may impair DNA binding activity of p65 in fibroblasts by sequestration or “squelching” in transcriptionally inactive p65-RelB complexes (69). LPS-tolerant THP-1 cells express p65 and RelB in the nucleus, and co-immunoprecipitation studies suggest that sequestration of p65 in the nucleus by RelB may play a role in silencing NFκB-dependent IL-1β transcription (44). However, our data indicate that RelB may act as a repressor at the promoter. This is the first study showing in vivo binding of RelB to the TNFα promoter as part of a gene silencing mechanism during endotoxin tolerance. Our results showed low level binding of RelB to the TNFα promoter in the basal state with an increase following LPS stimulation in tolerant cells. This increase in RelB binding did not quantitatively parallel the decrease in p65 binding. This also suggests that post-translational modification changes in RelB with or without a quantitative increase in levels of RelB may be required for repressor function. For example, RelB becomes phosphorylated on serine 368 (70) and regulated by dimerization (71). Other transcription factors and proteins that repress promoters by a process that is interdependent with H3(Lys3) methylation include TIF1α, p53, and retinoblastoma (Rb) protein (72–74). The repressor functions of these proteins are associated with histone H3 methylation and recruitment of HP1. Dissociation of HP1 from the repressed promoter upon histone H3 phosphorylation could promote the removal of co-repressors associated with it.

Of particular interest and relevant to our epigenetic findings and repression by RelB are studies of fibroblasts that constitutively silence TNFα even in response to stimulation with LPS (75). This silencing is associated with DNA methylation, and such cells constitutively express RelB (75, 76). When these silenced fibroblasts are rendered RelB−/−, they become responsive to LPS through augmentation of NF-κB activity and impairment of postinduction repression of NF-κB. It is possible that the RelB repressive function links both the disrupted recruitment of NF-κB p65 and biochemical processes that modify chromatin during LPS tolerance. It is also possible that RelB cooperates with other co-repressors such as histone deacetylases to mediate gene repression. Histone deacetylases reverse acetylation of histone (77), and overexpression of histone deacetylase isoforms has been shown to inhibit TNFα transcription in monocytes (32). In any event, RelB is both necessary and sufficient in regulating some acute proinflammatory genes in connection with chromatin remodeling (44).
In summary, our studies demonstrated marks of epigenetic silencing and disruption of NF-κB transcriptional processes during silencing of TNFα expression in endotoxin-tolerant THP-1 cells. Studies are underway to apply this model to silencing of blood leukocytes during severe systemic inflammatory states in humans, including trauma and sepsis. We provide a theoretical model of silencing in Fig. 6 that includes both histone H3 and NF-κB events. Histone H3(Ser10) phosphorylation and p65 binding correlate with activation of TNFα expression in LPS-responsive cells, whereas in LPS-tolerant cells, there is persistent methylation of H3(Lys4), HP1α binding, reduced phosphorylation of H3(Ser10), disruption of NF-κB p65 recruitment, and concomitant binding of transcriptional repressor RelB. Changes in chromatin structure may contribute to the sustained process of reprogramming of gene expression observed during severe systemic inflammation with endotoxin tolerance. We suggest that epigenetic controls play a major role in the process of inflammation whether it occurs locally or systemically.

REFERENCES

1. McCall, C. E., and Yozsa, B. K. (2007) Am. J. Respir. Crit. Care Med. 175, 763–767
2. Yozsa, B. K., Hu, J. Y., Cousart, S. L., and McCall, C. E. (2000) Shock 13, 236–243
3. Granowitz, E. V., Porat, R., Mier, J. W., Orencole, S. F., Kaplanski, G., Lynch, E. A., Ye, K., Vannier, E., Wolff, S. M., and Dinarello, C. A. (1993) J. Immunol. 151, 1637–1645
4. West, M. A., and Heagy, W. (2002) Crit. Care Med. 30, S64–S73
5. McCall, C. E., Grosso-Wilmoth, L. M., LaRue, K., Guzman, R. N., and Cousart, S. L. (1993) J. Clin. Investig. 91, 853–861
6. Munoz, C., Misset, B., Fitting, C., Berriot, J. P., Carlet, J., and Cavaillon, J. M. (1991) Eur. J. Immunol. 21, 2177–2184
7. LaRue, K. E., and McCall, C. E. (1994) J. Exp. Med. 180, 2269–2275
8. Li, B., Carey, M., and Workman, J. L. (2007) Cell 128, 707–719
9. Martin, C., and Zhang, Y. (2005) Nat. Rev. Mol. Cell. Biol. 6, 838–849
10. Struhl, K. (1999) Cell 98, 1–4
11. Ghosh, S., and Karin, M. (2002) Cell 109, (suppl.) S81–S96
12. Roth, S. Y., Denu, J. M., and Allis, C. D. (2001) Annu. Rev. Biochem. 70, 81–120
13. Berger, F., and Gaudin, V. (2003) Chromosome Res. 11, 277–304
14. Lacnner, M., and Jenuwein, T. (2002) Curr. Opin. Cell Biol. 14, 286–298
15. Cheung, P., Allis, C. D., and Sassone-Corsi, P. (2000) Cell 103, 263–271
16. Clayton, A. L., Rose, S., Barratt, M. J., and Mahadevan, L. C. (2000) EMBO J. 19, 3714–3726
17. Thomson, S., Clayton, A. L., Hazzalin, C. A., Rose, S., Barratt, M. J., and Mahadevan, L. C. (1999) EMBO J. 18, 4779–4793
18. Fischle, W., Wang, Y., and Allis, C. D. (2003) Nature 425, 475–479
19. Dormann, H. L., Tseng, R. S., Allis, C. D., Funabiki, H., and Fischle, W. (2006) Cell Cycle 5, 2842–2851
20. Natoli, G. (2006) FEBS Lett. 580, 2843–2849
21. Cheung, W. L., Briggs, S. D., and Allis, C. D. (2000) Curr. Opin. Cell Biol. 12, 326–333
22. Ogryzko, V. V., Schiltz, R. L., Russanova, V., Howard, B. H., and Nakatani, Y. (1996) Cell 87, 953–959
23. Sheppard, K. A., Rose, D. W., Haque, Z. K., Kurokawa, R., McNerney, E., Westin, S., Thanos, D., Rosenfeld, M. G., Glass, C. K., and Collins, T. (1999) Mol. Cell. Biol. 19, 6367–6378
24. Chen, L. F., Mu, Y., and Greene, W. C. (2002) EMBO J. 21, 6539–6548
25. Kiernan, R., Bres, V., Ng, R. W., Coudart, M. P., El Messaoudi, S., Sardet, C., Jin, D. Y., Emilian, S., and Benkirane, M. (2003) J. Biol. Chem. 278, 2758–2766
26. Saccani, S., Pantano, S., and Natoli, G. (2002) Nat. Immunol. 3, 69–75
27. Vandenh, B. W., Vermeulen, L., Delerive, P., De, B. K., Staels, B., and Hageman, G. (2003) Adv. Exp. Med. Biol. 544, 181–196
28. Chan, C., Li, L., McCall, C. E., and Yozsa, B. K. (2005) J. Immunol. 175, 461–468
29. Locksley, R. M., Killeen, N., and Lenardo, M. J. (2001) Cell 104, 487–501
30. Yao, J., Mackman, N., Edginton, T. S., and Fan, S. T. (1997) J. Biol. Chem. 272, 17795–17801
31. Han, J., Brown, T., and Beutler, B. (1990) J. Exp. Med. 171, 465–475
32. Miao, F., Gonzalez, I. G., Lanting, L., and Natarajan, R. (2004) J. Biol. Chem. 279, 18091–18097
33. Cavaillon, J. M., Adrie, C., Fitting, C., and Adib-Conquy, M. (2003) J. Endotoxin Res. 9, 101–107
34. Cavaillon, J. M., Adib-Conquy, M., Fitting, C., Adrie, C., and Payen, D. (2003) Scand. J. Infect. Dis. 35, 535–544
35. Mathison, J. C., Virca, G. D., Wolfson, E., Tobias, P. S., Glaser, K., and Ulevitch, R. J. (1990) J. Clin. Investig. 85, 1108–1118
36. Anracht, F., Garcia-Palomero, L., Lopez, J., Jimenez, M., Madero, R., Renart, J., Vazquez, J. J., and Montiel, C. (2000) Infect. Immun. 68, 1942–1945
37. Jenuwein, T., and Allis, C. D. (2001) Science 293, 1074–1080
38. Strahl, B. D., and Allis, C. D. (2000) Nature 403, 41–45
39. Jacobs, S. A., and Khorasanizadeh, S. (2002) Science 295, 2080–2083
40. Kuprash, D. V., Udalova, I. A., Turetskaya, R. L., Kwiatkowski, D., Rice, 792000
Silencing during Endotoxin Tolerance

N. R., and Nedospasov, S. A. (1999) J. Immunol. 162, 4045–4052

42. Udalova, I. A., Knight, J. C., Vidal, V., Nedospasov, S. A., and Kwiatkowski, D. (1998) J. Biol. Chem. 273, 21178–21186

43. Trede, N. S., Tytuszykova, A. V., Chatila, T., Goldfeld, A. E., and Gehr, R. S. (1995) J. Immunol. 155, 902–908

44. Yoo, B. K., Hu, J. Y., Cousart, S. L., Forrest, L. M., and McCall, C. E. (2006) J. Immunol. 177, 4080–4085

45. Barthel, R., and Goldfeld, A. E. (2003) J. Immunol. 171, 3612–3619

46. Boekhoudt, G. H., Guo, Z., Beresford, G. W., and Boss, J. M. (2003) J. Immunol. 170, 4139–4147

47. Fields, P. E., Kim, S. T., and Flavell, R. A. (2002) J. Immunol. 169, 647–650

48. Goriely, S., Demonte, D., Nizet, S., De, W. D., Willems, F., Goldman, M., and Van, L. C. (2003) Blood 101, 4894–4902

49. Rao, S., Gerondakis, S., Woltring, D., and Shannon, M. F. (2003) J. Immunol. 170, 3724–3731

50. Weinmann, A. S., Plevy, S. E., and Smale, S. T. (1999) Immunity 11, 665–675

51. Lachner, M., O’Carroll, D., Rea, S., Mechtler, K., and Jenuwein, T. (2001) Nature 410, 116–120

52. Snowden, A. W., Gregory, P. D., Case, C. C., and Pabo, C. O. (2002) Curr. Biol. 12, 2159–2166

53. Wissmann, M., Yin, N., Muller, J. M., Greschik, H., Fodor, B. D., Jenuwein, T., Vogler, C., Schneider, R., Gunther, T., Buettner, R., Metzger, E., and Schule, R. (2007) Nat. Cell Biol. 9, 347–353

54. Albig, W., Runge, D. M., Kratza, M., and Doenecke, D. (1998) FEBS Lett. 435, 245–250

55. Kouzarides, T. (2007) Cell 128, 693–705

56. Piacentini, L., Fanti, L., Berloco, M., Perrini, B., and Pimpinelli, S. (2003) J. Cell Biol. 161, 707–714

57. Cheung, P., Fanti, K. G., Cheung, W. L., Sassone-Corsi, P., Denu, J. M., and Allis, C. D. (2000) Mol. Cell 5, 905–915

58. Arnedt, V., Hanson, J. L., Cogswell, P. C., Steinbrecher, K. A., Strahl, B. D., and Baldwin, A. S. (2003) Nature 423, 659–663

59. Yamasato, Y., Verma, U. N., Prajapati, S., Kwak, Y. T., and Gaynor, R. B. (2003) Nature 423, 655–659

60. Park, G. Y., Wang, X., Hu, N., Pedchenko, T. V., Blackwell, T. S., and Christman, J. W. (2006) J. Biol. Chem. 281, 18684–18690

61. Freitag, M., and Selker, E. U. (2005) Curr. Opin. Genet. Dev. 15, 191–199

62. Fujita, N., Watanabe, S., Ichimura, T., Tsuruzoe, S., Shinkai, Y., Tachibana, M., Chiba, T., and Nakao, M. (2003) J. Biol. Chem. 278, 24132–24138

63. Hediger, F., and Gasser, S. M. (2006) Curr. Opin. Genet. Dev. 16, 143–150

64. Eisenberg, J. C., and Elgin, S. C. (2000) Mol. Cell. Biol. 20, 210–218

65. Verschure, P. J., van der Kraan, I., de Leeuw, W., van der Vlag, J., Carpen- ter, A. E., Belmont, A. S., and van Driel, R. (2005) Mol. Cell Biol. 25, 4552–4564

66. Liu, H., Sidirooulos, P., Song, G., Paglari, L. J., Birrer, M. J., Stein, B., Anrather, J., and Pope, R. M. (2000) J. Immunol. 164, 4277–4285

67. Tsai, E. Y., Yie, J., Thanos, D., and Goldfeld, A. E. (1996) Mol. Cell. Biol. 16, 5232–5244

68. Bohrer, H., Qiu, F., Zimmermann, T., Zhang, Y., Illmer, T., Mannel, D., Bottiger, B. W., Stern, D. M., Walldherr, R., Saeher, H. D., Ziegler, R., Bierhaus, A., Martin, E., and Nawroth, P. P. (1997) J. Clin. Investig. 100, 972–985

69. Marienfeld, R., May, M. J., Berberich, I., Serfling, E., Ghosh, S., and Neu- mann, M. (2003) J. Biol. Chem. 278, 19852–19860

70. Maier, H. J., Marienfeld, R., Wirth, T., and Banmann, B. (2003) J. Biol. Chem. 278, 39242–39250

71. Saccani, S., Pantano, S., and Natali, G. (2003) Mol. Cell 11, 1563–1574

72. Le, D. B., Nielsen, A. L., Garnier, J. M., Ichinose, H., Jeanmougin, F., Llos- son, R., and Chambon, P. (1996) EMBO J. 15, 6701–6715

73. Esteve, P. O., Chin, H. G., and Pradhan, S. (2007) J. Biol. Chem. 282, 2615–2625

74. Nielsen, S. J., Schneider, R., Bauer, U. M., Bannister, A. J., Morrison, A., O’Carroll, D., Firestein, R., Cleary, M., Jenuwein, T., Herrera, R. E., and Kouzarides, T. (2001) Nature 412, 561–565

75. Xia, Y., Chen, S., Wang, Y., Mackman, N., Ku, G., Lo, D., and Feng, L. (1999) Mol. Cell. Biol. 19, 7688–7696

76. Krays, V., Thompson, P., and Butler, B. (1993) J. Exp. Med. 177, 1383–1390

77. Berger, S. L. (2002) Genet. Dev. 12, 142–148