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Chromatin-Specific Remodeling by HMGB1 and Linker Histone H1 Silences Proinflammatory Genes during Endotoxin Tolerance▽

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Epigenetic silencing of tumor necrosis factor alpha (TNF-α) and interleukin 1β (IL-1β) transcription occurs in blood leukocytes of animals and humans after the initiation of severe systemic inflammation (SSI). We previously reported that the epigenetic signature requires induction of NF-κB factor RelB, which directs histone H3K9 dimethylation, disrupts assembly of transcription activator NF-κB p65, and induces a sustained switch from the euchromatin to heterochromatin. Here, we report the novel findings that intracellular high mobility group box 1 protein (HMGB1) and nucleosome linker histone H1 protein are necessary components of endotoxin-mediated silencing of TNF-α in THP-1 human promonocytes. HMGB1 binds the TNF-α promoter during transcription silencing and promotes assembly of the repressor RelB. Depletion of HMGB1 by small interfering RNA results in dissociation of RelB from the promoter and partially restores TNF-α transcription. Histone H1, which typically displaces HMGB1 from nucleosomal DNA, also binds concomitantly with HMGB1 to the heterochromatin of the silenced TNF-α promoter. Combined knockdown of HMGB1 and H1 restores binding of the transcriptionally active NF-κB p65 and reestablishes TNF-α mRNA levels. Chromatin reimmunoprecipitation experiments demonstrate that HMGB1 and H1 are likely recruited to TNF-α sequences independently and that their binding correlates with histone H3K9 dimethylation, as inhibition of histone methylation blocks HMGB1 and H1 binding. Moreover, HMGB1- and H1-mediated chromatin modifications are gene specific during endotoxin silencing in that they also bind and repress acute proinflammatory IL-1β, while no binding nor repression of antiinflammatory IκBα is observed. Finally, we find that H1 and HMGB1 bind to the TNF-α a promoter in human leukocytes obtained from patients with SSI. We conclude proinflammatory HMGB1 and structural nucleosome linker H1 couple as a component of the epigenetic complex that silences acute proinflammatory TNF-α during the assembly of heterochromatin in the SSI phenotype.

Severe systemic inflammation (SSI), as caused by sepsis and other disseminated acute inflammatory processes, is associated with transient induction of acute proinflammatory mediators, followed by their sustained repression (reviewed in reference 29). In contrast, expression of other sets of genes is sustained during this reprogramming process (54). The altered gene expression pathway is flexible and differentially affects many gene sets with clinically relevant signatures. The gene-specific reprogramming includes acute proinflammatory tumor necrosis factor alpha (TNF-α) and interleukin 1β (IL-1β) (repressed), antiinflammatory IL-1 receptor antagonist (increased), late proinflammatory high mobility group box protein 1 (HMGB1) (increased), and antimicrobial defensins (increased) (15, 28, 29, 54). Gene programming that occurs during SSI of humans and animals can be faithfully modeled in vitro by generating a state of lipopolysaccharide endotoxin (LPS) tolerance (24, 49, 55). The transcription silencing phase of the proinflammatory genes develops rapidly (3 to 5 h) after the initial activation that generates a "cytokine storm." The reprogramming profile is common to SSI in animals (27) and humans (9) and is observed in blood neutrophils (28) and monocytes (31). The role of HMGB1 as a persistently produced inflammatory mediator has received considerable attention for SSI (1, 2, 30, 33, 51).

HMGB proteins are non-histone chromatin-associated molecules that bend DNA and bind preferentially to distorted DNA structures (reviewed in reference 47). They contain two DNA-binding HMG boxes and a long acidic C-terminal tail. While the structure of the three members of the HMGB family is highly conserved and their biochemical properties are indistinguishable, they exhibit a different expression pattern (30). HMGB1 is ubiquitously expressed, whereas HMGB2 and HMGB3 expression is restricted to embryonic development and to lymphoid organs (30). HMGB1 and -2 are highly similar but distinct in the length of the acidic tail and may act primarily as architectural facilitators in the assembly of nucleoprotein complexes and the maintenance of chromatin. HMGB1 can support transcriptional activation (8, 47). Nuclear HMGB proteins generally function by overcoming the rigidity of DNA and thereby promoting the formation of transcription complexes containing tightly bent DNA (11). Because the HMGB domain lacks selectivity to DNA sequences, targeting HMGB proteins to promoter sequences likely depends on the presence of se-

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quence-specific DNA-binding regulatory proteins in the transcription complex (47). This notion is supported by in vitro studies showing that HMGB1 and -2 enhance the binding of various transcription factors like Oct-1 and -2 (56), p53 (20), Rel family proteins (13, 38), and steroid hormone receptors (6) to their DNA binding sites. Also, HMGB1 interacts with NF-κB p65 to activate the melanoma inhibitory gene promoter (38). These studies support that HMGB1 and -2 not only serve as nonspecific DNA-binding architectural proteins but also can contribute to gene-specific transcriptional regulation.

As a late mediator of inflammation that is released from cells during SSI, extracellular HMGB1 binds with high affinity to the receptor for advanced glycation end (RAGE) products (21) and signals through Toll-like receptors 2 and 4 (34, 35) to activate innate immune cells (2). Under physiological conditions, HMGB1 shuttles between the chromatin bound and unbound state (4). In necrotic cells, HMGB1 is released from the nucleus. In contrast, it is retained by condensed chromatin in the nucleus of apoptotic cells (43). A monoclonal antibody (Ab) against HMGB1 protects against sepsis and increases survival in animal models of sepsis (50, 52). Mice administered with HMGB1 have increased levels of serum TNF-α (2). It is counterintuitive that plasma levels of HMGB1 coincide with transcription silencing of TNF-α and IL-1β (29).

We discovered that transcription silencing of acute proinflammatory genes during human SSI involves an epigenetic process characterized by the formation of silent facultative heterochromatin (10, 14). The heterochromatin is marked by increased methylation of histone H3 on lysine 9 (H3K9), binding of heterochromatin protein 1 (HP1), and binding the NF-κB transcriptional repressor RelB to the proximal promoter sequences (10, 14, 53). The euchromatin response of phosphorylation of histone H3 on serine 10 is lost during silencing, indicating a shift in the "histone code."

Here, we report a novel interaction between HMGB1 and the linker histone H1 as essential components of the chromatin transcription silencing process for TNF-α in the SSI phenotype, using both the THP1 cell model and blood leukocytes from humans with SSI. The repressor nature of HMGB1 and histone H1 is coupled to methylation of H3K9 and promoter binding of the transcription factor and repressor, RelB. The repressive function of HMGB1 and H1 is specific to silenced genes, as their knockdown did not affect the actively transcribed IκBα gene during endotoxin-mediated silencing. We conclude that HMGB1 has dual functions as an extracellular proinflammatory mediator and an intracellular antiinflammatory nuclear chromatin modulator.

MATERIALS AND METHODS

Cell culture. The human promonocytic cells, THP-1, obtained from the American Type Culture Collection, were maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 10% fetal bovine serum (HyClone, Logan, UT) in a humidified incubator with 5% CO_2 at 37°C. For induction of LPS tolerance (24), cells were incubated for 16 h with 1 μg/ml of gram-negative bacterial LPS (*Escherichia coli* O111:B4 [Sigma, St. Louis, MO], which acts solely through the Toll-like receptor 4). LPS-silenced and LPS-responsive (healthy) cells were washed with minimal medium, cultured at 1×10^6 cells/ml, and stimulated with 1 μg/ml LPS for the indicated times.

Isolation of human blood leukocytes. Blood polymorphonuclear leukocytes (PMN) were obtained by layering whole blood over Isolymph (Gallard-Schlesinger Industries, Carle Place, NY) and allowing it to settle for 40 min, after which the white blood cell (WBC)-rich plasma was removed and centrifuged at $100 \times g$ for 8 min. We adjust the cell differentials between healthy participants and participants with SSI by further isolating PMN and resuspending based on PMN contributions to the cell types of SSI blood leukocytes. PMN are pelleted by resuspending the WBC in phosphate-buffered saline, layering them over Isolymph (4 ml WBC over 3 ml Isolymph), and centrifuging at $400 \times g$ for 30 min. Red blood cells were removed by hypotonic lysis using 3 parts distilled H_2O for 20 s followed by 1 part of 3.6% NaCl. Pelleted PMN were resuspended in RPMI complete medium at a concentration of 5×10^6 cells/ml, producing a 98% pure population. Cells were stimulated with 100 ng/ml LPS for the indicated amounts of time.

RNA interference. Cells were plated at 0.5×10^6 cells/ml 1 day before transfection. Transfection with pools of control, HMGB1- or H1.1- plus H1.4-specific small interfering RNAs (siRNA) (Santa Cruz Biotechnology, Santa Cruz, CA) was performed by electroporation using Nucleofector (amaxa, Gaithersburg, MD) and 5 μl (0.5 μM) siRNA in 100 μl transfection medium. Immediately after transfection, cells were transferred to culture medium at 0.5×10^6 cells/ml and left unstimulated or stimulated with 1 μg/ml LPS to induce tolerance. After 36 h, cells were harvested, washed with minimal medium, and stimulated for 3 h with 1 μg/ml LPS.

RNA transcripts. Expression of TNF-α, IL-1β, and IκBα was evaluated by quantitative real-time PCR. Total RNA was isolated using the RNA STAT-60 extraction kit, according to the manufacturer's protocol (Tel-Test, Friendswood, TX). Two micrograms of RNA was reverse transcribed to cDNA in a 25-μl volume containing 0.2 μM deoxynucleoside triphosphate (dNTP), 2.5 μM oligo(dT), 5 mM $MgCl_2$, and 0.25 U/μl of murine leukemia reverse transcriptase (Applied Biosystems). The RT reaction was incubated for 1 h at 42°C and 5 min at 99°C. The PCR analysis was performed using 5 μl cDNA and gene-specific predesigned TaqMan primer/probe sets (Applied Biosystems). PCR conditions were as described above. Sample data were normalized to GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA and are presented as fold change relative to mRNA from unstimulated cells (assigned onefold).

Western blot analysis. Nuclear proteins were extracted by incubating cells on ice for 15 min in a buffer containing 10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM EDTA, 20 mM NaF, 1 mM $Na_4P_2O_7$, 1 mM Na_3VO_4, 0.5 mM dithiothreitol, 0.1% Triton X-100, and 1× protease inhibitor cocktail. Nuclei were pelleted by centrifugation at 5,000 rpm for 10 min at 4°C and then resuspended in lysis buffer (1% NP-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate [SDS]) and incubated on ice for 30 min. Extracts were cleared by centrifugation, and the protein concentration was determined. Whole-cell extracts were prepared using the same nuclei lysis buffer. Equal amounts (50 μg) of protein were separated on SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Pierce, Rockford, IL). Membranes were blocked and probed overnight at 4°C with appropriate dilutions of primary Abs against HMGB1 (R&D Systems, Minneapolis, MN) or H1 (Santa Cruz Biotechnology). This was followed by incubation with appropriate horseradish peroxidase-conjugated secondary Abs (Santa Cruz Biotechnology). Proteins were visualized using the enhanced chemiluminescence detection system (Pierce). Blots were stripped and reprobed with control Ab.

ChIP. A chromatin immunoprecipitation (ChIP) assay was performed to assess in vivo DNA-protein interactions at the TNF-α promoter, using a ChIP assay kit according to the manufacturer's instructions (Active Motif, Carlsbad, CA). Briefly, cells were harvested and fixed in 1% (vol/vol) formaldehyde in minimal culture medium for 10 min at room temperature (to cross-link proteins with DNA). After washing with cold phosphate-buffered saline, cells were lysed in 1% SDS for 30 min on ice. The lysates were sonicated to shear DNA using a Branson 250 sonicator (two 15-s pulses at 40% power in an ice bath, with 1 min between each pulse). These shearing conditions generate DNA fragments ranging in size from 500 to 1,000 bp. Chromatin solution was precleared with protein G-coated magnetic beads for 2 h at 4°C. Ten microliters of the chromatin solution was reserved as the "input" sample. The remaining chromatin was immunoprecipitated overnight at 4°C with 3 μg Ab specific to HMGB1 (R&D Systems), linker histone H1, NF-κB p65, RelB, HP1, NAP1, immunoglobulin G (IgG) as a negative control (Santa Cruz Biotechnology), or dimethylated histone H3 lysine 9 (H3K9me2; Upstate Biotechnology, Lake Placid, NY). The chromatin-Ab complexes captured on the beads were washed several times and then eluted in 50 μl elution buffer. The immunoprecipitated and "input" sample cross-links were reversed by incubation for 2.5 h at 65°C. After treatment with proteinase K at for 1 h at 37°C, the reaction was stopped and the resulting DNA was stored at −20°C until analyzed by standard and real-time PCR as described below.

For the ChIP reimmunoprecipitation experiment, chromatin was first immunoprecipitated with the primary Ab (anti-H1, HMGB1, or anti-RelB) and

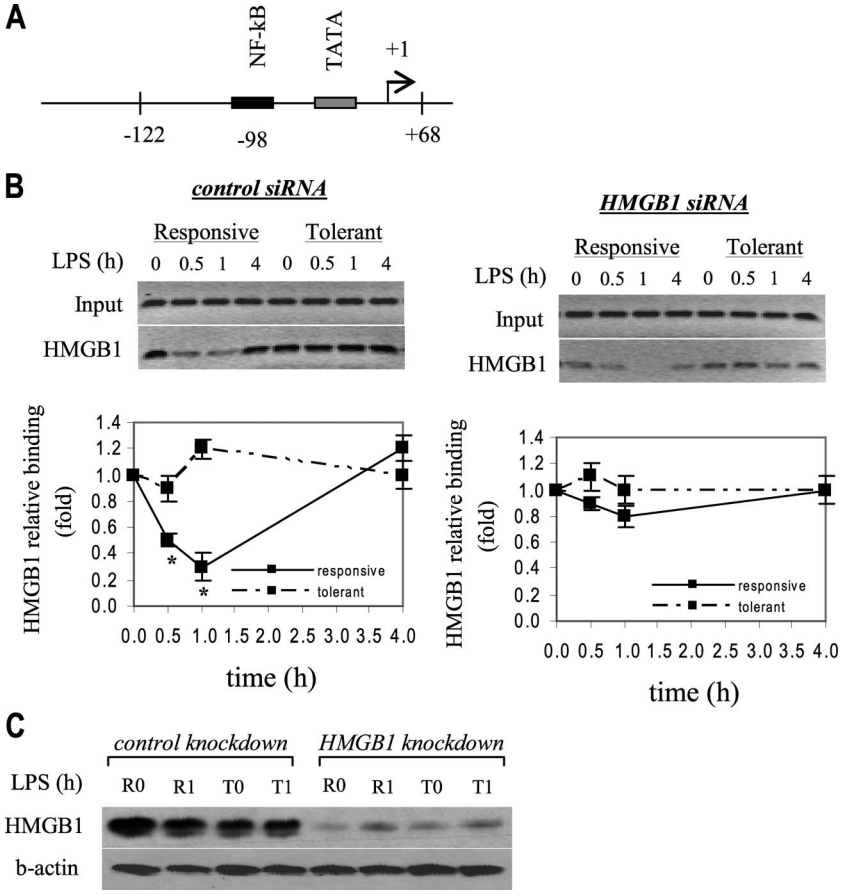

FIG. 1. HMGB1 binds the TNF-α promoter during transcription silencing. (A) A schematic diagram of the human TNF-α proximal promoter region showing the locations of the NF-κB binding site and the primers used for PCR analysis. (B) ChIP analysis of HMGB1 binding to the TNF-α promoter. THP-1 cells were transfected with nonspecific or HMGB1-specific siRNA and left unstimulated (responsive) or stimulated for 36 h with 1 μg/ml LPS (to induce tolerance). Cells were then washed and left unstimulated (0 h) or stimulated with 1 μg/ml LPS for the indicated amounts of time. Cross-linked chromatin was isolated and immunoprecipitated with HMGB1 Ab. DNA recovered without immunoprecipitation (input) was used as an internal control. The enrichment of TNF-α promoter sequences in the immunoprecipitated DNA (which reflects the amount of protein binding in vivo) was measured by standard (top) and real-time (bottom) PCR. Sample data were normalized to input DNA and are presented as change relative to unstimulated cells (0 h) (assigned onefold). Data show the mean ± SEM from three experiments. *, $P < 0.05$. (C) Western blot analysis of HMGB1 protein in the nucleus. Cells were transfected and treated as described for panel B. Nuclear proteins were extracted at 0 h and 1 h after LPS stimulation and then probed with HMGB1 Ab. Blots were stripped and reprobed with control Ab. R, responsive; T, tolerant.

washed. Complexes were then eluted from the primary immunoprecipitates by incubation with 10 mM dithiothreitol at 37°C for 25 min, diluted in immunoprecipitation buffer, and reimmunoprecipited with the second Ab (ReIP). The remaining ChIP procedures were followed to reverse the cross-links and recover the DNA, as described above.

Semiquantitative PCR. PCR analysis was performed in a 50-μl volume containing 5 μl ChIP DNA, 1 μM of each primer, 2 mM $MgCl_2$, 0.2 μM dNTP, and 0.04 U/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°C, 58°C, 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 1.2% ethidium bromide-stained agarose gel, and images were captured using a Quantity One imager (Bio-Rad, Hercules, CA). The primers used in the PCR were designed to amplify a sequence in the human TNF-α proximal promoter region containing the κB3 site at −98 bp relative to the transcription start site (17) and were TNF-α forward (5′-TACCGCTTCCT CCAGATGAG-3′) and reverse (5′-TGCTGGCTGGGTGTGCCAA-3′). The primers used to amplify IκBα promoter region containing the κB1 site at −96 bp were IκBα forward (5′-AGCAGAGGACGAAGCCAGTTCT-3′) and reverse (5′-GACTGCTGTGGGCTCTGCAG-3′). The primers used for IL-1β were described previously (10).

Quantitative real-time PCR. Immunoprecipitated DNA was analyzed using PCR primers specific to the TNF-α promoter between −122 and +68 and a fluorescently labeled internal probe. Primer and probe sequences are as follows:

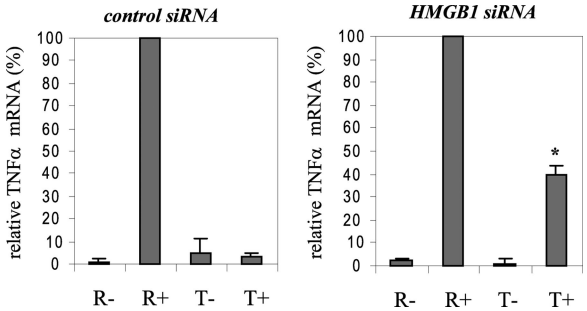

FIG. 2. HMGB1 knockdown partially restores TNF-α transcription in silenced cells. THP-1 cells were transfected and left unstimulated (responsive) or stimulated with LPS. After 36 h, responsive (R) and silenced (T) cells were washed and then left unstimulated (−) or stimulated (+) with 1 μg/ml LPS for 3 h. Total RNA was extracted and analyzed by real-time PCR. Sample data were normalized to GAPDH mRNA and are presented as percentage of change relative to the mRNA level from LPS-responsive (R+) cells (set as 100%). Data are the mean ± SEM from three experiments. *, $P < 0.05$ (T+ versus T−).

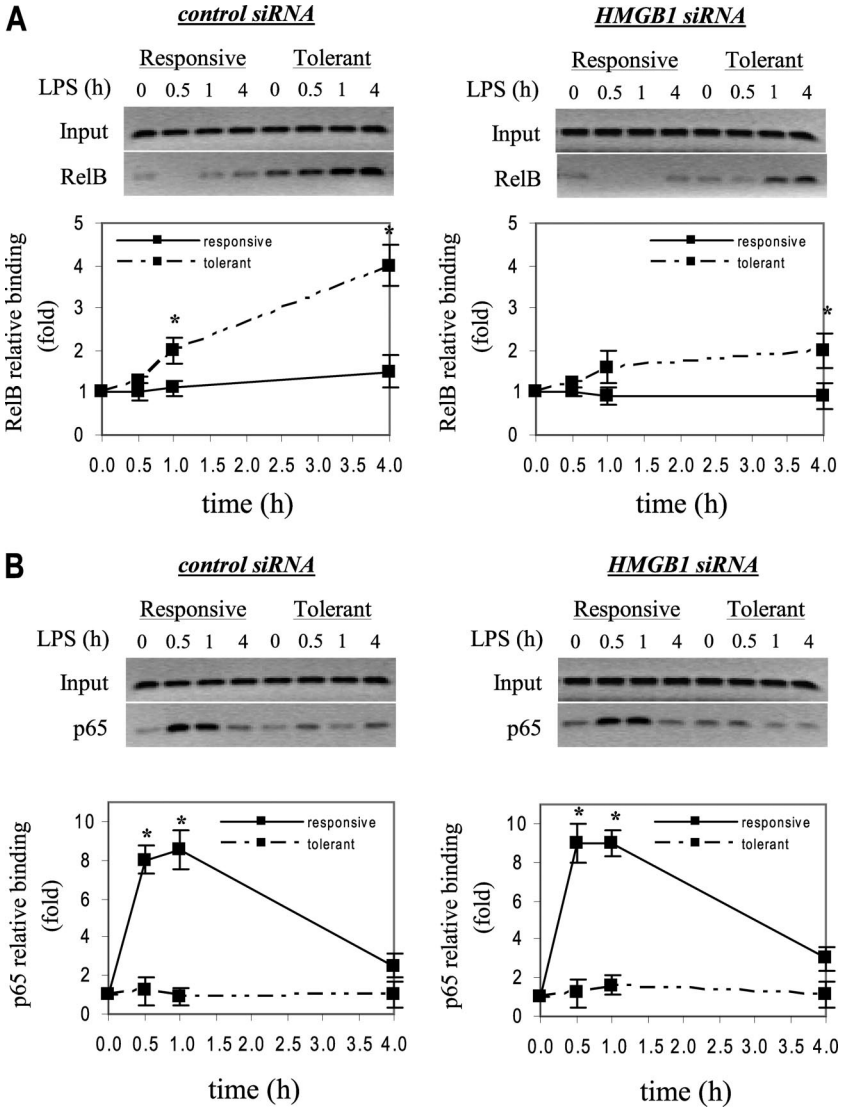

FIG. 3. Loss of HMGB1 binding at the TNF-α promoter decreases RelB but not p65 binding. Cells were transfected and treated as described in the legend to Fig. 1. Chromatin was isolated and immunoprecipitated with RelB or p65 Ab. ChIP DNA was analyzed by standard (top) and real-time (bottom) PCR. Real-time PCR data are the mean ± SEM from three experiments. *, $P < 0.05$.

TNF-α forward, 5′-TACCGCTTCCTCCAGATGAG-3′; TNF-α reverse, 5′-TG CTGGCTGGGTGTGCCAA-3′; and probe, 5′-FAM-CTTGGTGGAGAAAC C-3′-6-carboxytetramethylrhodamine (Applied Biosystems, Foster City, CA). Samples were analyzed in duplicates. The PCR (25 μl) contained 5 μl ChIP DNA, 12.5 μl of 2× TaqMan universal master mix containing DNA polymerase and dNTP, 300 nM of each primer, and 100 nM internal probe. The PCR conditions were as follows: 2 min at 50°C, 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 the 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). Relative enrichment of DNA sequences was calculated by normalizing averaged cycle threshold values to the input DNA. These values are presented as fold change relative to DNA from unstimulated cells (0 h).

Statistical analysis. Data were analyzed by Microsoft Excel 2003 and are presented as the mean ± the standard error of the mean (SEM) of three independent experiments. Student's *t* test was used to determine significant differences between groups. *P* values of <0.05 were considered statistically significant.

RESULTS

HMGB1 binds TNF-α promoter during endotoxin-mediated silencing and its depletion partially restores TNF-α transcription. We first showed that HMGB1 protein was equally expressed in the nuclei of responsive and tolerant cells (our preliminary results and Fig. 1C). We next examined HMGB1 binding to the TNF-α promoter in vivo, using a ChIP assay. We found HMGB1 not bound during TNF-α transcription activation in responsive THP-1 cells (our preliminary results and Fig. 1B). HMGB1 bound the promoter at low levels in unstimulated responsive cells, which dissociated after LPS stimulation and rebound at 4 h poststimulation, which coincides with the early inception time of endotoxin unresponsiveness. Surprisingly, we observed that HMGB1 bound the promoter throughout the course of LPS stimulation in silenced cells. These

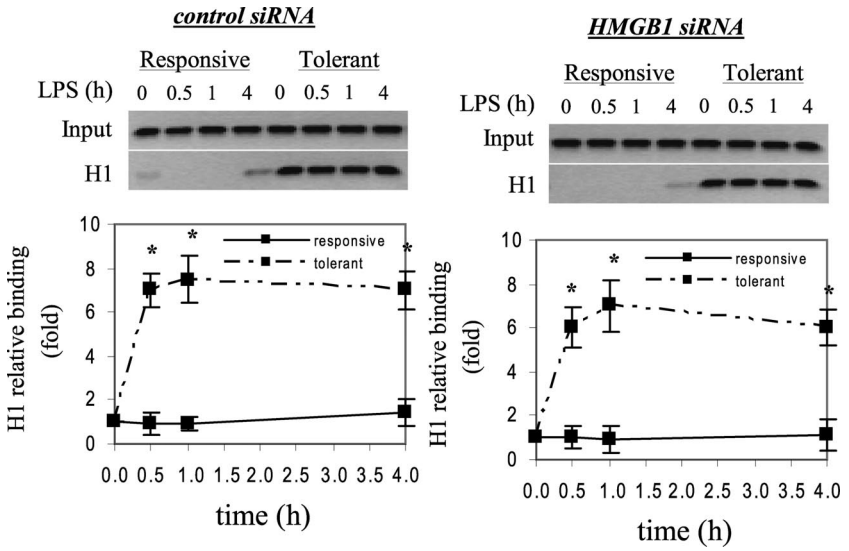

FIG. 4. HMGB1 knockdown does not affect H1 binding to the TNF-α promoter. Cells were transfected with nonspecific siRNA or a mixture of H1.1- plus H1.4-specific siRNA and treated as described above. Chromatin was isolated and immunoprecipitated with H1 Ab, and ChIP DNA was analyzed by PCR. Real-time PCR data are the mean ± SEM from three experiments. *, $P < 0.05$.

results suggested that, at the molecular level, HMGB1 does not contribute to LPS-induced expression of TNF-α in endotoxin-responsive cells and might participate in transcription silencing.

To examine whether HMGB1 binding in tolerant cells plays a role in TNF-α transcription repression, we blocked HMGB1 expression by siRNA interference using a pool of HMGB1-specific siRNAs. First, we measured HMGB1 binding to TNF-α promoter sequences in ChIP DNA by standard and real-time PCR. Figure 1B shows a significant decrease in HMGB1 binding after the knockdown with HMGB1-specific, but not with control nonspecific, siRNA. We also observed a marked decrease in the levels of HMGB1 protein expression in the nucleus (Fig. 1C).

Next, we measured TNF-α mRNA levels after HMGB1 knockdown. As shown in Fig. 2, TNF-α transcripts were partially restored in endotoxin-silenced cells upon LPS stimulation. Together, the data presented in Fig. 1 and 2 indicate that HMGB1 plays a role in transcription silencing of the TNF-α gene during endotoxin silencing.

HMGB1 participates in transcription silencing of TNF-α by stabilizing the repressor RelB complex at the promoter sequences. We reported that binding of the repressive NF-κB protein RelB and displacement of the active NF-κB p65 from the TNF-α and IL-1β promoters during endotoxin tolerance of THP-1 cells are required for transcription silencing (14, 53). We reasoned that HMGB1 may displace RelB from the promoter during restoration of TNF-α mRNA production, resulting in binding of transcription activator p65 (10, 14). Therefore, we assessed RelB binding after HMGB1 knockdown. Figure 3A shows a significant decrease in RelB binding, which is enriched at the promoter in silenced cells (14). The decrease in RelB binding in tolerant cells was not accompanied by p65 recruitment to the NF-κB site at −95 (which is the focus of this study), as we expected (Fig. 3B). These results suggest that HMGB1 mediates TNF-α transcription silencing, at least partly, by stabilizing RelB protein binding at the promoter.

HMGB1 cooperates with the linker histone H1 to silence TNF-α transcription during endotoxin-mediated silencing. HMGB1 binds to its cognate site in chromatin, the linker DNA between nucleosomes, and facilitates gene-specific transcription factor binding by mediating the initial steps of chromatin activation through nucleosomal sliding (5, 7, 48). In addition, biochemical studies have supported competition between HMGB1 and H1. For example, HMGB1 binds to nucleosomes at sites overlapping those recognized by H1, suggesting that HMGB1 may compete or substitute for H1 in binding the linker DNA (32). Unfolded (active) chromatin is characterized by relative depletion of H1 and enrichment of HMGB1 on linker DNA (19). The notion that H1 may act as a global repressor of transcription (18, 42) together with our unexpected finding that HMGB1 binds the TNF-α promoter at silent heterochromatin during endotoxin tolerance raised the possibility that HMGB1 may interact, rather than compete, with H1 to silence TNF-α transcription. Therefore, we assessed possible interactions between HMGB1 and H1.

We first measured H1 binding to TNF-α promoter by analyzing ChIP DNA immunoprecipitated with Ab that recognizes total H1. We found that H1 binds to the promoter in silenced cells only (our preliminary data and Fig. 4). Since HMGB1 knockdown partially restored TNF-α transcription (Fig. 2), we next tested whether depletion of HMGB1 affects H1 binding. As shown in Fig. 4, loss of HMGB1 expression did not affect H1 binding in silenced cells, indicating that HMGB1 does not direct the recruitment or binding of H1. In the reverse experiment, we knocked down H1 and assayed HMGB1 binding. Transfection of a mixture of H1 siRNAs into tolerant cells caused a significant decrease in H1 protein expression (Fig. 5C) and binding to the TNF-α promoter (Fig. 5A). As shown in Fig. 5B, H1 knockdown significantly decreased HMGB1

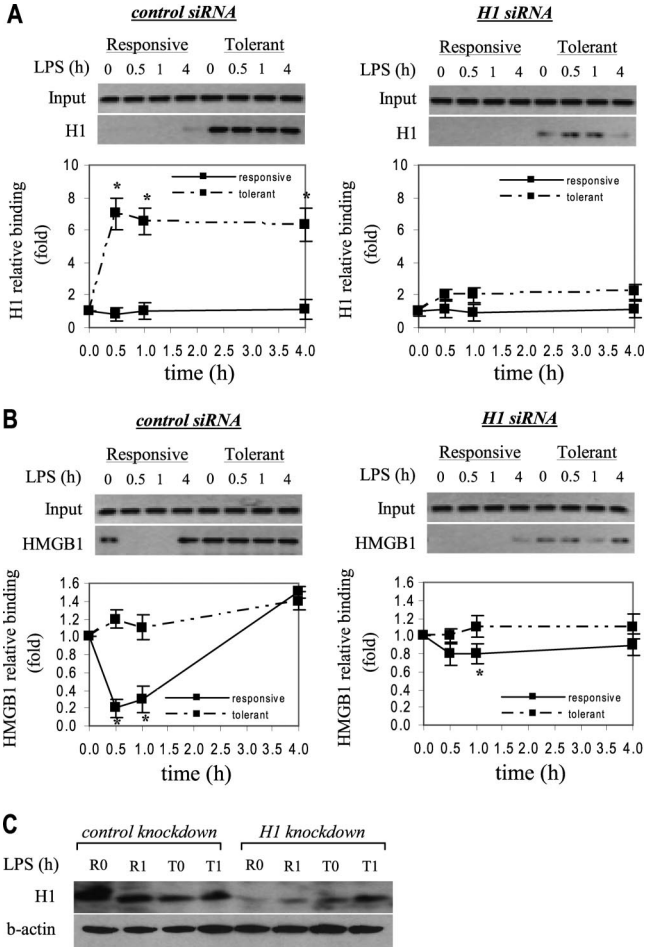

FIG. 5. H1 knockdown causes a marked decrease in HMGB1 binding to TNF-α promoter. Cells were transfected and treated as described above. Chromatin was immunoprecipitated with H1 (A) or HMGB1 (B) Ab. Real-time PCR data are the mean ± SEM from three experiments. *, $P < 0.05$. (C) Western blotting of H1 nuclear expression after H1 knockdown. Nuclear proteins were extracted at the indicated times and then probed with H1 Ab. R, responsive; T, tolerant.

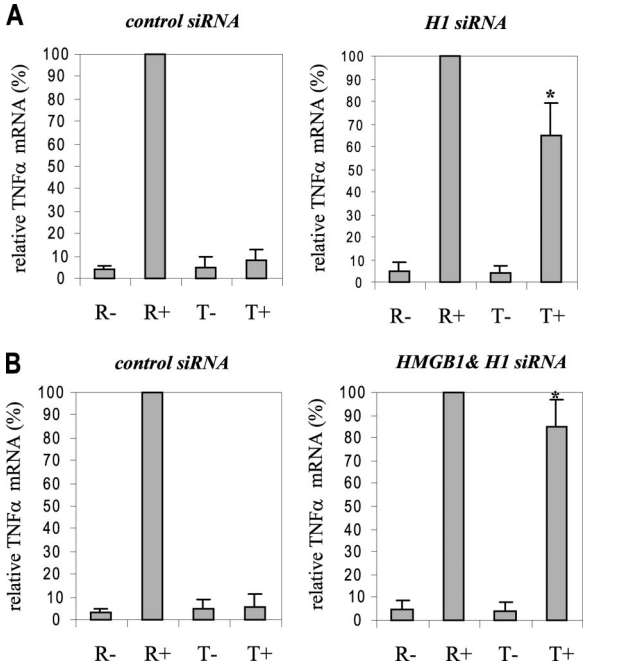

FIG. 6. Loss of H1 binding to promoter sequences markedly restores TNF-α expression, while simultaneous loss of HMGB1 and H1 binding releases repression and completely restores TNF-α transcripts. Cells were transfected with H1 siRNA alone (A) or a mixture of HMGB1 and H1 siRNAs (B) and left unstimulated or stimulated for 36 h (to induce tolerance). Unstimulated (responsive) and stimulated (silenced) cells were washed and left unstimulated (−) or stimulated (+) with 1 μg/ml LPS for 3 h. RNA was extracted and analyzed by real-time PCR. Data are the mean ± SEM from three experiments. R, responsive; T, tolerant; *, $P < 0.05$ (T+ versus T−).

binding in tolerant cells, suggesting the involvement of H1 in HMGB1 binding to the TNF-α promoter.

The data presented above showed that HMGB1 binding was dependent on H1 and that HMGB1 knockdown partially reactivated TNF-α transcription. To measure the effect of H1 knockdown on TNF-α transcription, we blocked H1 expression by siRNA transfection. Figure 6A shows that TNF-α transcription is markedly increased in tolerant cells after H1 knockdown. The increase in TNF-α mRNA level was higher than its level seen after HMGB1 knockdown (Fig. 2). Next, we measured TNF-α mRNA expression after combined HMGB1 and H1 knockdown. The results (Fig. 6B) show that depletion of HMGB1 and H1 in tolerant cells reactivates TNF-α promoter and restores transcription to a level close to that seen in endotoxin-responsive cells. The data presented in Fig. 2 and 6 support that HMGB1 and H1 cooperate to silence TNF-α expression during endotoxin tolerance, with H1 being the more potent repressor.

HMGB1 and H1 silence TNF-α transcription by regulating transcription factor binding. We reported that p65 binds to and activates the TNF-α promoter in endotoxin-responsive cells and that the loss of RelB binding restores TNF-α transcription and p65 binding in tolerant cells (14). Here, we determined the effect of combined HMGB1 and H1 knockdown on RelB and p65 binding. The results (Fig. 7) showed a significant decrease in RelB binding that was accompanied by a marked increase in p65 recruitment to the promoter in tolerant cells, suggesting that HMGB1 and H1 cooperatively interact and couple to RelB to silence TNF-α transcription by promoting RelB binding in tolerant cells. Removal of the repressor complex permits p65 binding, which has accumulated in the nucleus during silencing (14, 53), resulting in a shift of facultative chromatin to the transcriptionally active euchromatic state.

HMGB1 and H1 are independently recruited to the TNF-α promoter and form a protein complex with RelB. The results presented above suggested that H1 may participate in recruiting HMGB1 to the TNF-α promoter, because depletion of H1 resulted in loss of HMGB1 binding. To assess possible interactions between HMGB1, H1, and RelB at the TNF-α promoter, we performed a chromatin reimmunoprecipitation assay in which cross-linked chromatin was isolated from responsive and silenced cells at 1 h after LPS stimulation. Chromatin was first immunoprecipitated with H1 Ab (ChIP).

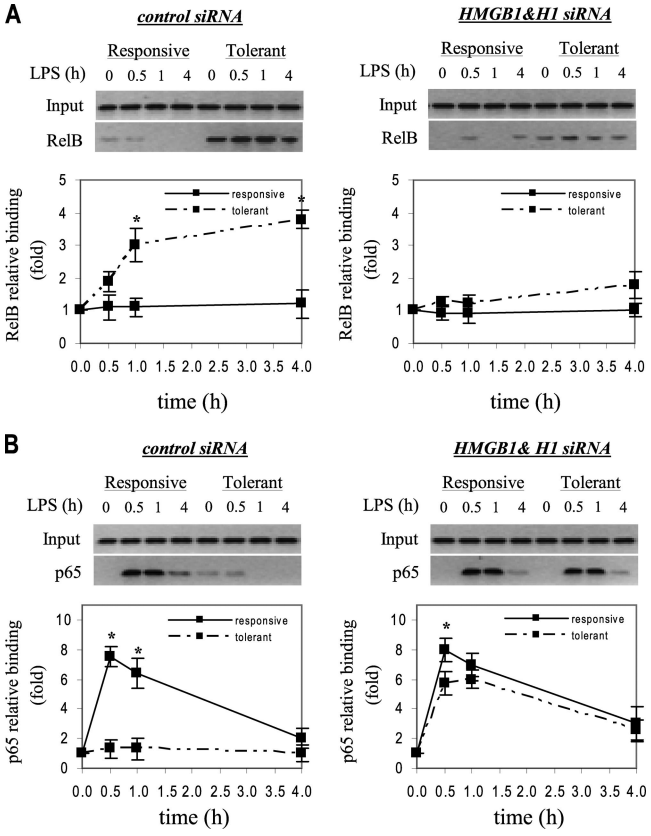

FIG. 7. HMGB1 and H1 knockdown prevents RelB binding and restores p65 binding in silenced cells. Cells were transfected and stimulated as described above. Responsive and silenced cells were then restimulated for the indicated amounts of time. Chromatin was immunoprecipitated with RelB (A) or p65 (B) Ab. ChIP DNA was analyzed by PCR. Data are the mean ± SEM from three experiments. *, $P < 0.05$.

Immunoprecipitates were then eluted and reimmunoprecipitated with HMGB1 or RelB Ab (ReIP). We did not detect HMGB1 and H1 binding in responsive cells (as described above). In silenced cells, we detected TNF-α promoter sequences in the second immunoprecipitate (ReIP) pulled with RelB but not with HMGB1 Ab (Fig. 8A, middle). In the inverse experiment, we used RelB as the primary Ab and then reprobed the immunoprecipitated complex from tolerant cells with Ab against H1 or HMGB1 as the secondary Ab. As shown in Fig. 8A (right), TNF-α DNA was detected in both H1 and HMGB1 immunoprecipitates. When we used anti-HMGB1 as primary Ab and then reimmunoprecipitated the complex with H1 or RelB Ab we detected DNA in RelB immunoprecipitates only, indicating that HMGB1 binds directly to RelB (data not shown). Together, these results suggest that H1 is not directly responsible for recruiting HMGB1 and that HMGB1 does not directly interact with H1. The results also suggest that both H1 and HMGB1 are part of the RelB repressor complex.

Histone methylation and silent heterochromatin assembly mediate HMGB1 and H1 binding to TNF-α promoter sequences during transcription silencing. Both HMGB1 and H1 lack DNA-sequence recognition selectivity (1, 18). Thus, their targeting to the TNF-α promoter must be achieved by a different mechanism(s). In addition, we have shown that core histone H3K9 methylation (14) and DNA methylation (unpublished observation) at the TNF-α promoter contribute to heterochromatin formation and transcription silencing. We therefore investigated the effect of this epigenetic event on targeting HMGB1 and H1 binding during endotoxin tolerance. Silenced cells (which show HMGB1 and H1 binding) were incubated with 4 mM of the methyltransferase inhibitor 5′-deoxy-5′-methylthioadenosine (MTA), which is known to inhibit methyltransferase activity by blocking methyl group exchange (26, 45). Cells were incubated for 6 h with MTA while LPS was added for the last hour. We found HMGB1 and H1 dissociated from the promoter (Fig. 8B). This binding pattern was not changed when LPS was added. The decrease in binding was not due to toxic effects by MTA because cell viability was about 90% after 6 h in MTA. Also, we have found that H3K9 dimethylation is directed by the histone methyltransferase G9a, which also binds to the TNF-α promoter during endotoxin tolerance (unpublished observation). We next blocked H3K9 dimethylation by incubating silenced cells for 6 h with 5 μM of the G9a-specific inhibitor BIX 012194 (22) and then stimulating with LPS for 1 h. Cell viability was more than 85% by the end of incubation. As shown in Fig. 8C, we observed a significant decrease in H3K9 dimethylation accompanied by a marked reduction in HMGB1 and H1 binding. We also detected a decrease in the HP1 binding, which binds TNF-α promoter in silenced cells (14). The results, as presented in Fig. 8B and C, clearly demonstrate that HMGB1 and H1 targeting and binding to the TNF-α promoter during endotoxin tolerance depend on epigenetic modifications of TNF-α promoter nucleosome through H3K9 methylation and silent heterochromatin assembly.

HMGB1 and H1 contribute to silencing IL-1β, but not IκBα, expression in endotoxin-tolerant cells. The results described above clearly implicated HMGB1 and H1 in the silencing of TNF-α transcription during endotoxin tolerance. To test whether such a silencing role is gene specific, we investigated the effects of HMGB1 and H1 knockdown on the expression proinflammatory IL-1β and IκBα genes. The IL-1β gene is silenced (10, 53), while IκBα expression is elevated during endotoxin tolerance (29 and our unpublished observations). First, we measured HMGB1 and H1 binding to the IL-1β and IκBα promoters. As shown in Fig. 9, we detected significant amounts of HMGB1 and H1 bound to the IL-1β promoter in tolerant cells. In the meantime, we did not detect any significant binding of either protein to the IκBα promoter. Next, we examined the effect of HMGB1 and H1 knockdown on IL-1β and IκBα expression. Figure 10 shows that the IL-1β mRNA level increased significantly in tolerant cells (Fig. 10A), while there were no obvious effects on the elevated expression of IκBα (Fig. 10C).

Our studies showed that H3K9 and HP1 played a role in silencing IL-1β (10 and our unpublished observations) and TNF-α (14) expression in endotoxin-tolerant cells. In addition, our results showed that HMGB1 and H1 played a role in TNF-α silencing by coupling to H3K9 methylation and HP1 binding. To determine the effects of these heterochromatin marks on HMGB1 and H1 binding to the IL-1β promoter, we incubated tolerant cells with the methyltransferase inhibitor MTA, which inhibits histone methylation and HP1 binding (14,

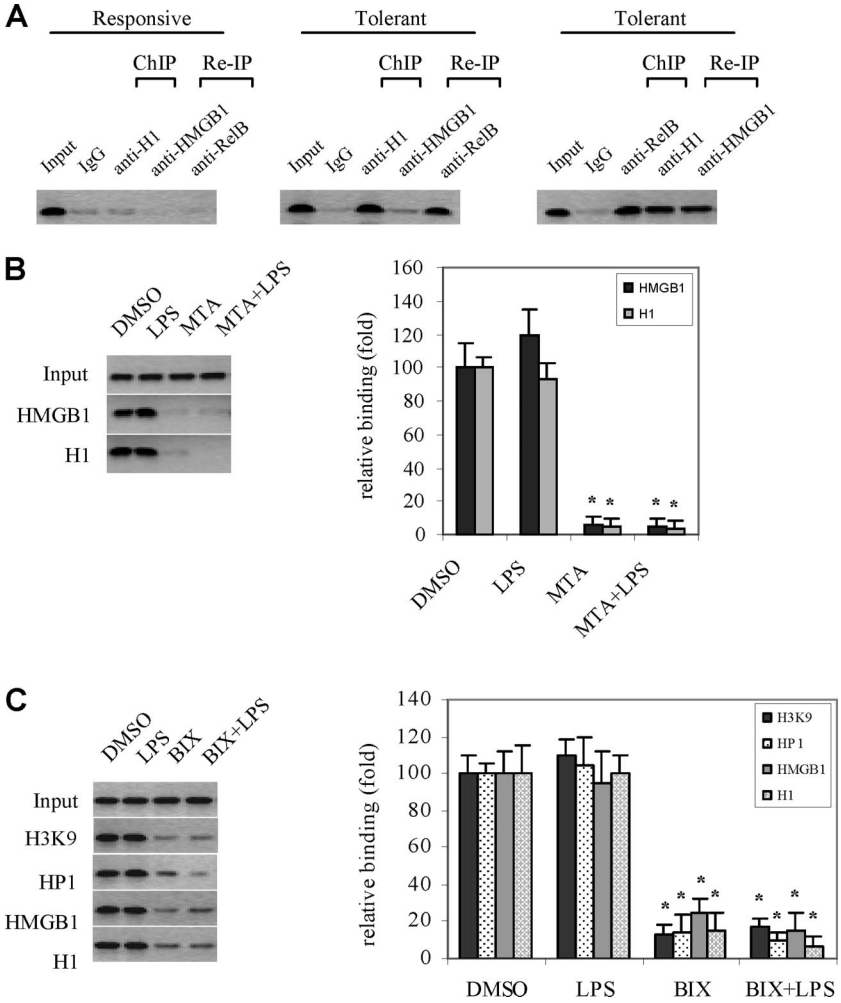

FIG. 8. (A) HMGB1 and H1 are recruited to the TNF-α promoter independently and form a complex with RelB. Responsive and silenced cells were stimulated with 1 μg/ml LPS for 1 h (at which time HMGB1 and H1 bind in silenced cells). Chromatin was first immunoprecipitated with H1 Ab (ChIP). Immunoprecipitates were eluted and reimmunoprecipitated with HMGB1 or RelB Ab (ReIP). An IgG-immunoprecipitated sample is shown as a negative control. HMGB1 and H1 form a complex with RelB. Chromatin was immunoprecipitated with RelB Ab, eluted, and then reimmunoprecipitated with H1 or HMGB1 Ab. (B) Inhibiting histone methylation prevents HMGB1 and H1 binding during tolerance. Silenced cells were incubated for 6 h with 4 mM of the methyltransferase inhibitor MTA. LPS was added for the last hour. Chromatin was isolated and immunoprecipitated with HMGB1 or H1 Ab. ChIP DNA was analyzed by standard and real-time (right) PCR. (C) Dimethyl H3K9 recruits HMGB1 and H1 to TNF-α promoter during tolerance. Silenced cells were incubated for 6 h with 5 μM of the G9a methyltransferase inhibitor BIX 012194. LPS was then added for the last hour. Chromatin was immunoprecipitated with dimethyl H3K9, HMGB1, H1, or HP1 Ab. Results from standard and real-time PCR analyses are shown. Real-time PCR data are the mean ± SEM from three experiments. DMSO, dimethyl sulfoxide; *, $P < 0.05$ (compared with LPS alone).

26, 45). As shown in Fig. 10B, we did not detect HMGB1, H1, H3K9, and HP1 binding after LPS stimulation of tolerant cells treated with MTA, suggesting that HMGB1 and H1 binding were dependent on H3K9 methylation and heterochromatin assembly. In addition, we did not detect HMGB1 and H1 binding at the transcriptionally competent IκBα promoter in tolerant cells after LPS stimulation (Fig. 10D). Together, the results presented in Fig. 9 and 10 demonstrate that HMGB1 and H1 are involved in the transcription silencing of IL-1β but have no effects on IκBα expression during endotoxin tolerance. These gene-specific results also suggest that the silencing effects on IL-1β expression are mediated in part by epigenetic modifications involving H3K9 methylation and heterochromatin formation.

HMGB1 and H1 bind to the TNF-α promoter in blood leukocytes from sepsis patients. Our previous studies support that the THP-1 cell model closely mimics the reprogramming phenotype observed in human blood leukocytes during SSI (14, 53, 54). Gene silencing in SSI has profound clinical implications. To test the proof of concept relative to HMGB1 and H1 as potential contributors to silencing in SSI, we investigated blood leukocytes obtained from healthy patients and patients with SSI, as previously described (53). Over 90% of these cells are neutrophils. To assess the presence of heterochromatin at the TNF-α promoter, we used the silencing H3K9 dimethylation mark. Figure 11 shows the loss of H3K9 dimethylation following LPS activation of normal blood leukocytes with subsequent remethylation. In contrast, H3K9 dimethylation is

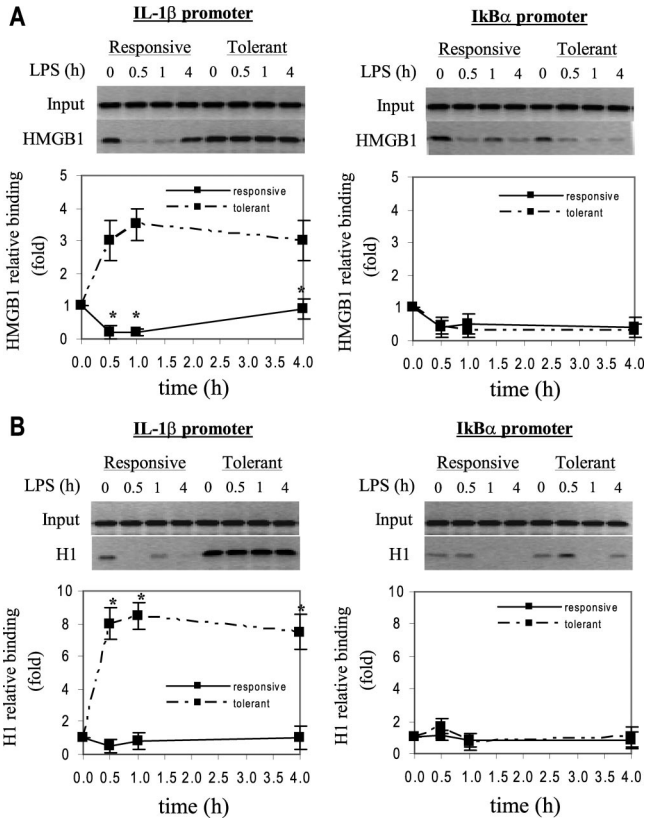

FIG. 9. HMGB1 and H1 bind to the promoter of repressed IL-1β but not the transcribed IκBα gene in silenced cells. (A) HMGB1 binding. Cross-linked chromatin was isolated and immunoprecipitated with HMGB1 Ab and then assayed for the presence of IL-1β and IκBα sequences as described above. (B) H1 binding. Chromatin was immunoprecipitated with H1 Ab and then assayed for the presence of IL-1β and IκBα sequences. Real-time PCR data are the mean ± SEM from three experiments. *, $P < 0.05$.

constitutively present in SSI leukocytes. SSI blood leukocytes also show consistent binding of both HMGB1 and H1 to the TNF-α promoter, supporting their contribution to epigenetic silencing in this inflammatory disease.

DISCUSSION

Herein, we show that HMGB1 and H1 linker histone are required elements of gene-specific transcription silencing during epigenetic reprogramming of gene expression in the SSI phenotype. This is the first study to directly implicate HMGB1 and H1 in proinflammatory gene silencing through an epigenetic-mediated transcription regulatory mechanism. Furthermore, this process is reversible, supporting the facultative nature of the chromatin. Data supporting this paradigm include the following: (i) both HMGB1 and H1 bind in vivo to TNF-α DNA, as assessed by ChIP; (ii) removal of HMGB1 alone partially restores transcription, and knockdown of both almost totally restores transcription; (iii) HMGB1 and H1 couple to the repressor function of RelB, as supported by the loss of RelB promoter binding and gain of p65 binding in the reversed phenotype; (iv) inhibition of dimethylation of the repressive chromatin mark H3K9 leads to dissociation of HMGB1 and H1 promoter binding, indicating a close link to the "histone code"; (v) HMGB1 is not required for H1 binding, while H1 binding is obligate for HMGB1 binding, although the two mediators bind independently to the heterochromatin as part of the RelB repressor complex; (vi) HMGB1 and H1 chromatin modifications are promoter selective during endotoxin silencing in that they also bind and repress acute proinflammatory IL-1β, while no binding nor repression of antiinflammatory IκBα is observed. H1 and HMGB1 bind to the TNF-α promoter of blood leukocytes obtained from humans with SSI, while cells from healthy human subjects do not bind these two proteins.

The dual function of HMGB1 as an architectural chromatin-binding protein and a proinflammatory signal highlights both its importance and complex role as a mediator of innate immune responses (30, 47). While its role as a proinflammatory mediator in extending and sustaining the inflammatory response to promote SSI is clear (1, 51), it is less apparent how HMGB1 acts as structural component of the chromatin and transcriptional machinery in regulating gene expression. The present study clearly shows that HMGB1 contributes to epigenetic-mediated silencing of TNF-α and IL-1β during endotoxin silencing, a gene expression signature faithful to that of humans and animals with SSI. The precise mechanism is unclear, but posttranslational modifications are candidates.

Posttranslational modifications and protein-protein interactions are mechanisms for targeting HMGB1 and H1 to nucleosomal DNA sites. In the case of HMGB1, hyperacetylation or ribosylation (40) is linked to its release from the nucleus (5). In addition, the C-terminal tail of HMGB1 plays a role in transcription stimulation by interacting with the core histones, including H3 and H2A-H2B dimers (47, 48). H1 is also covalently modified. Multiple phosphorylation sites on H1 occur and may weaken its binding to chromatin, promoting chromatin decondensation/activation and enabling access to transcription factors (16, 18, 41). Lysine methylation on H1 is implicated in transcriptional repression (23). Also, H1.4 (the major H1 variant) is methylated at lysine 26, which plays a role in transcription repression (16, 23). This methylation mark facilitates heterochromatin assembly by targeting HP1 to chromatin (12). We conducted preliminary mass spectrometry analysis and observed multiple phosphorylation, acetylation, and methylation on HMGB1 (data not shown). These modifications were similar in both endotoxin-responsive (in which HMGB1 did not bind TNF-α sequences) and silenced cells. We also detected lysine 26 methylation on H1.4 at similar levels in both responsive and silenced cells. In addition, Western blot analysis suggested that protein expression level of HMGB1 and H1 was not a rate-limiting factor in their binding to TNF-α promoter in silenced cells as opposed to responsive cells because protein levels did not vary in both normal and silenced cells. Therefore, protein expression or posttranslational modifications of HMGB1 and H1 as rate-limiting factors are, at this time, unidentified in our system.

We reported that posttranslational modifications with dimethylation of core histone H3K9 play an essential role in heterochromatin assembly and transcription silencing of TNF-α (14). Our current experiments clearly support that H3 methylation is required and may precede the targeting of both HMGB1 and H1 to TNF-α promoter and that this process

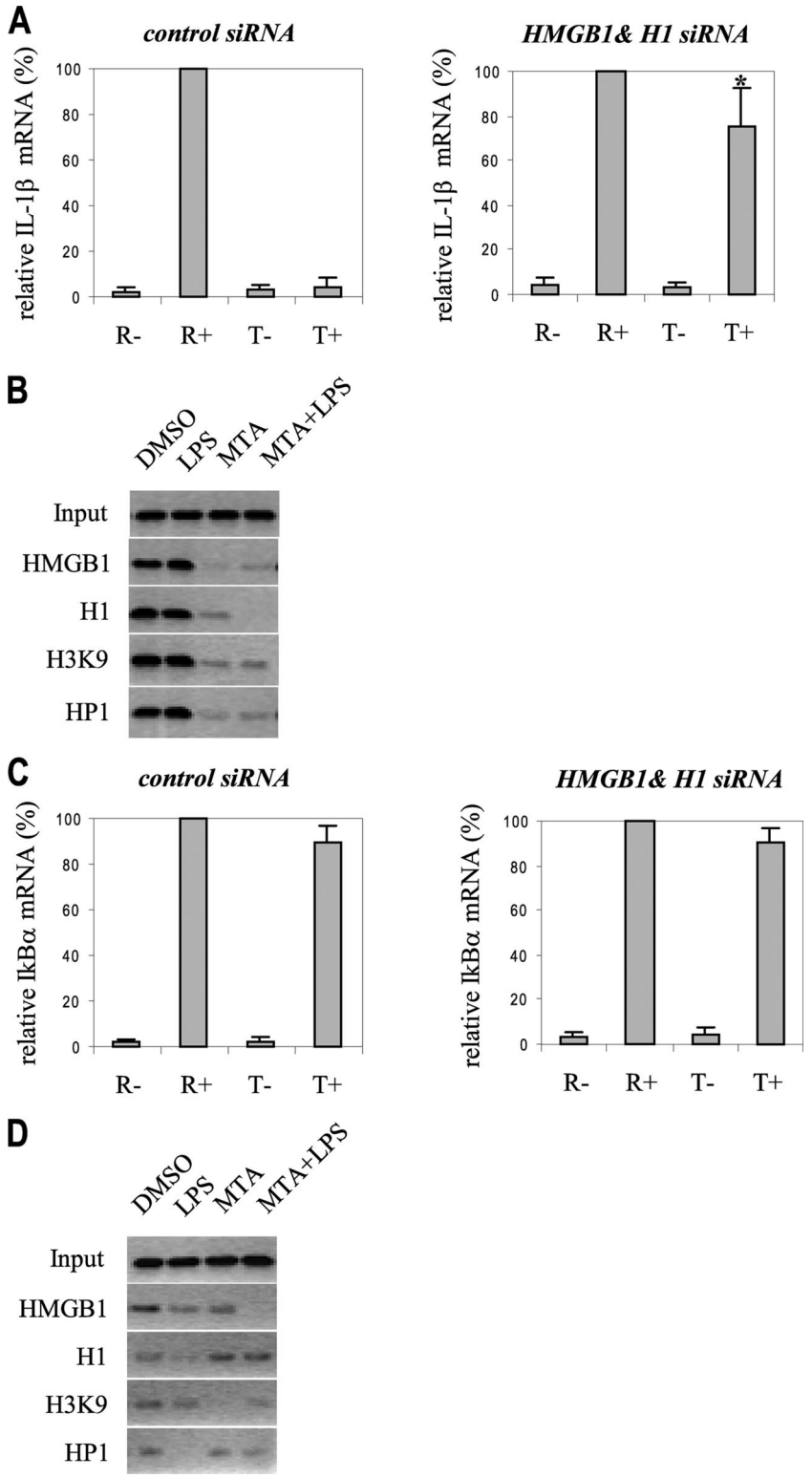

FIG. 10. HMGB1 and H1 knockdown reactivates IL-1β expression in silenced cells. Cells were transfected with a mixture of HMGB1 and H1 siRNAs (A and C) and left unstimulated or stimulated for 36 h with 1 μg/ml LPS (to induce tolerance). Unstimulated (responsive) and stimulated (tolerant) cells were washed and then left unstimulated (−) or stimulated (+) with 1 μg/ml LPS for 3 h. RNA was isolated and analyzed for IL-1β (A) and IκBα (C) expression by real-time PCR. Data are the mean ± SEM from three experiments. R, responsive; T, tolerant; *, $P < 0.05$ (T+ versus T−). Inhibition of histone H3 methylation prevents HMGB1, H1, and HP1 binding at the IL-1β promoter in silenced cells. Silenced cells were incubated for 6 h with 4 mM of the histone methyltransferase inhibitor MTA. LPS was added for the last hour. Chromatin was isolated and immunoprecipitated with HMGB1, H1, H3K9, and HP1. DNA was analyzed by standard PCR for the presence of IL-1β (B) and IκBα (D) promoter sequences. The results shown are representative of two experiments. DMSO, dimethyl sulfoxide.

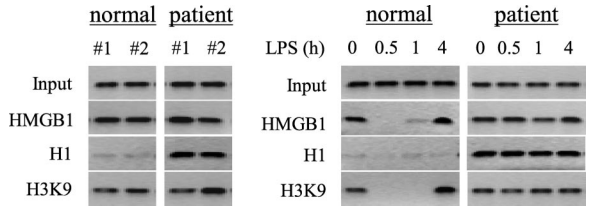

FIG. 11. HMGB1 and H1 binding to TNF-α promoter in human blood leukocytes. Leukocytes (mostly neutrophils in patients) were isolated from healthy subjects (normal) and sepsis patients (patient). Normal cell preparations were adjusted to a comparable percentage of neutrophils (90 to 95%). Cells were stimulated with 0.1 μg/ml for the indicated times. Chromatin was immunoprecipitated with HMGB1, H1, or H3K9 Ab. Standard PCR results of unstimulated leukocytes from two healthy participants and two patients (left) and stimulated leukocytes from one healthy subject and a third patient with SSI (right) are shown.

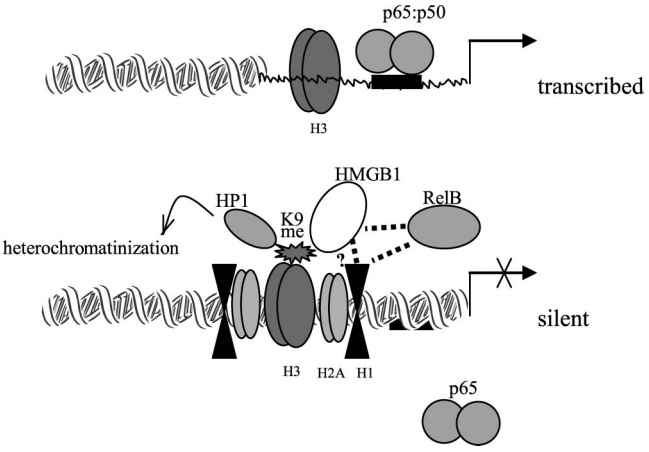

FIG. 12. A model depicting the putative role of histone chaperones HMGB1 and H1 in chromatin remodeling and transcriptional silencing during endotoxin tolerance. Endotoxin initiates an epigenetic signal to methylate H3K9 of euchromatin domain and recruits HP1 which would help assemble and close the chromatin structure. H1 and HMGB1 are then recruited where H1 "locks" the heterochromatin and assists HMGB1 in maintaining the transcriptional repressor RelB. RelB may interact directly or indirectly with HMGB1 through an undefined protein(s). Loss of H1 would lead to disassembly of heterochromatin and loss of HMGB1 and RelB, resulting in restoration of the competent endotoxin responsive state through access to p65 and other transcription activators and coactivators. K9me, methylated lysine 9.

requires binding and activity of the histone methyltransferase G9a (unpublished observation). In this study, chemically blocking H3 dimethylation on lysine 9 (H3K9) prevented H1 and HMGB1 binding in silenced cells, further confirming the role of epigenetic signals in chromatin remodeling and HMGB1 and H1 assembly at proinflammatory nucleosomes in silenced cells. The gene selectivity of this process may have implications for developing specific antiinflammatory therapies for SSI.

Our findings support that HMGB1 and H1 concurrently bind TNF-α linker DNA in silenced cells, since HMGB1 binding depended on the presence of H1. In addition, loss of HMGB1 expression by siRNA transfection did not affect H1 binding, suggesting that H1 is proximal to the binding or stabilizing of HMGB1 complex. However, our coimmunoprecipitation experiments suggested that HMGB1 and H1 were independently recruited to the TNF-α promoter and that together they may form a protein complex with transcriptionally repressive RelB. We also showed that HMGB1 knockdown resulted in the loss of RelB binding. In addition, our previous experiments showed that inhibiting RelB expression restored both TNF-α and IL-1β mRNA levels in tolerant cells (14, 53). Thus, it is possible that the endotoxin-induced epigenetic signals allow changes in chromatin configuration that favor the assembly of a silencing complex in which HMGB1 and H1 provide architectural foundation for stabilizing and promoting the repressive function of RelB. HMGB1 has been shown to enhance the binding of various transcription factors, including p65 and steroid hormone receptors, to their cognate DNA binding sites (6, 56).

H1 limits transcription factor accessibility by inhibiting nucleosome sliding or replacement and thus facilitating chromatin condensation and transcriptional repression by masking the initiation site (3, 25, 46). Our results showed that combined HMGB1 and H1 knockdown eliminated RelB, restored p65 binding, and reactivated TNF-α transcription. Temporary release of H1 from TNF-α nucleosomal DNA may allow chromatin decondensation, which would reopen the gate for gene-specific transcription factor to bind. One such opening factor could be the nucleosome assembly protein NAP1, which is implicated in the regulation of transcription factor binding to chromatin and augmenting the activity of many p300 target genes, including p53 and E2F (39, 44). We find that removal of HMGB1 and reduction in RelB binding results in binding of NAP1 (which we detected in responsive cells only) to the TNF-α promoter in silenced cells (data not shown). This might facilitate the removal of H2A-H2B core histone dimers, resulting in nucleosome sliding or replacement and exposing the enhancer (36, 37, 44). Although NAP1 may target p65 to TNF-α promoter, we could not detect p65 binding after depletion of HMGB1 protein and partial reversal of phenotypes. p65 binding was detected only after removal of both HMGB1 and H1. A trivial explanation for this is that presence of H1 limits a facilitory step for p65 assembly via NAP1. In addition, there is an upstream κB binding site at −213 in the TNF-α promoter. Therefore, it is also possible that binding of p65 to this site may be responsible, at least partly, for the partial reactivation of TNF-α expression in tolerant cells seen after HMGB1 knockdown, without p65 rebinding to the proximal κB site.

This study also revealed the selective nature of HMGB1 and H1 silencing effects on proinflammatory gene expression during endotoxin tolerance. Our experiments showed HMGB1 and H1 binding to and silencing of IL-1β, but not IκBα, transcription. IκBα mRNA and protein levels remain elevated in tolerant cells, where H3K9 methylation is not apparent at its promoter (our unpublished observations). On the other hand, H3K9 methylation and RelB binding are required for the IL-1β transcription silencing during endotoxin tolerance (10, 53). Thus, it appears that epigenetic modifications on chromatin play a role in promoting the repressive function of chromatin-associate proteins on proinflammatory genes in humans during endotoxin tolerance. An elegant study of the gene-specific control of inflammation by chromatin modifications in

the murine model of endotoxin tolerance supports this paradigm (15).

Our discovery adds yet another component to the highly complex nature of the repressor complex of the SSI innate immunity regulation, which collectively involves transcription machinery, DNA methylation, histone regulation, and nucleosome positioning, all of which occur in a spatial and temporal context. More layering of this important rubric of nature likely will emerge, since other mutually contributory processes were reported in the murine model of reprogramming for SSI (15). Thus, the mechanisms responsible for the gene-specific feature of SSI are emerging. Finally, our findings in blood leukocytes of humans with SSI support the HMGB1/H1 repressor paradigm as clinically relevant.

In conclusion, our theoretical paradigm for the epigenetics of transcription silencing in the SSI phenotype is depicted in Fig. 12. This study added the nuclear structural proteins HMGB1 and histone linker H1 to the transcriptional repressor factors and the histone nucleosomal posttranslational modifications. We suggest that the role of H1 and HMGB1 is to "lock" or facilitate condensation of chromatin that masks positive *cis* elements by nucleosomal positioning. The silencing process likely affects defined sets of genes across the genome. Since the condensation of chromatin is reversible, it becomes possible to test the clinical effects of restoring the competency of this element of the innate immune response in the highly lethal state of SSI.

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