

Regulation of NF- κ B Circuitry by a Component of the Nucleosome Remodeling and Deacetylase Complex Controls Inflammatory Response Homeostasis^{*[5]}

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The MTA1 coregulator (**metastatic tumor antigen 1**), a component of the nucleosome remodeling and deacetylase (NuRD) complex, has been intimately linked with human cancer, but its role in inflammatory responses remains unknown. Here,

we demonstrated that MTA1 is a target of inflammation and that its overexpression in macrophages promotes the NF- κ B signaling pathway, leading to increased expression of NF- κ B target genes. In contrast, depletion of MTA1 in macrophages inhibits NF- κ B signaling and reduces the expression of NF- κ B target genes. Thus, MTA1 is a novel component of the NF- κ B signaling pathway that regulates the inflammatory response. MTA1 is a member of the MTA family of ubiquitously expressed chromatin modifiers, the MTA family is well known for the integral role in NuRD complexes that is to modify DNA accessibility for cofactors (12–14). MTA1, the first identified member of the MTA family of genes, regulates cellular pathways by associating and modifying the acetylation status of the target gene chromatin. MTA1 is widely up-regulated in a wide variety of human tumors (14–16). MTA1 regulates transcription of its targets by modifying acetylation around the target chromatin and cofactor accessibility to the target DNA. MTA1 functions not only as a transcriptional repressor of estrogen receptor α (17) but also as a transcriptional activator on certain promoters, such as the BCAS3 (**b**reast **c**ancer-**a**mplified **s**equences **3**) (18) and Pax5 transcription (19). Although MTA1 has been linked intimately with human cancer and widely is regarded as a potential master coregulator, the role of MTA1 in inflammatory responses remains unrecognized and delineated here. For the first time,

WITHDRAWN
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This article has been withdrawn by the authors. Upon becoming aware of concerns raised regarding errors with respect to Fig. 4B, the authors are withdrawing the paper and apologize for these errors. The senior author states that the experiments and the final assembly of Fig. 4B were performed by specific co-authors from his laboratory. The authors state that the potential issues raised with Fig. 4B do not affect the scientific conclusions of this work.

induced by LPS, revealing that MTA1 protects mice from deregulated host inflammatory response. These findings reveal a previously unrecognized, critical homeostatic role of MTA1, both as a target and as a component of the NF- κ B circuitry, in the regulation of inflammatory responses.

The inflammatory response is an integral part of the host defense mechanism against microbial agents that demands rapid and coordinated control of multiple inflammatory genes in immune cells, including macrophages. Early inflammatory response to microbial products such as lipopolysaccharide (LPS)³ activates the NF- κ B signaling pathway (1); once acti-

ing family of ubiquitously expressed chromatin modifiers, the MTA family is well known for the integral role in NuRD complexes that is to modify DNA accessibility for cofactors (12–14). MTA1, the first identified member of the MTA family of genes, regulates cellular pathways by associating and modifying the acetylation status of the target gene chromatin. MTA1 is widely up-regulated in a wide variety of human tumors (14–16). MTA1 regulates transcription of its targets by modifying acetylation around the target chromatin and cofactor accessibility to the target DNA. MTA1 functions not only as a transcriptional repressor of estrogen receptor α (17) but also as a transcriptional activator on certain promoters, such as the BCAS3 (**b**reast **c**ancer-**a**mplified **s**equences **3**) (18) and Pax5 transcription (19). Although MTA1 has been linked intimately with human cancer and widely is regarded as a potential master coregulator, the role of MTA1 in inflammatory responses remains unrecognized and delineated here. For the first time,

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[5] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Tables 1–4 and Figs. 1–3.

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³ The abbreviations used are: LPS, lipopolysaccharide; MTA, metastatic tumor antigen; NuRD, nucleosome remodeling and deacetylase; q-PCR, quantitative real-time PCR; siRNA, small interfering RNA; ChIP, chromatin immunoprecipitation; pol, polymerase; EMSA, electrophoretic mobility shift assay; IL, interleukin; TNF, tumor necrosis factor; luc, luciferase; MEF, mouse embryonic fibroblast; HDAC, histone deacetylase; MIP2, macrophage inflammatory protein-2; IKK, I κ B kinase.

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we provide evidence that MTA1 plays a critical homeostatic role in inflammatory responses both as a target and as a component of the NF- κ B circuitry.

EXPERIMENTAL PROCEDURES

Antibodies and Cell Culture—Antibodies against MTA1 (A300-280A), MTA2 (A300-395A), MTA3 (A300-160A), and RNA polymerase II (pol II) (A300-653A) were purchased from Bethyl Laboratories (Montgomery, TX); and HDAC2 (catalog no. sc-9959), NF- κ B p65 (p65) (catalog no. sc-372), phospho-NF- κ B p65 (catalog no. sc-33020), NF- κ B p50 (catalog no. sc-7178), phospho-NF- κ B p50 (catalog no. sc-33022-R), and NF- κ B p65 (catalog no. 286-H) X (catalog no. sc-7151 X) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Phospho-I κ B α (catalog no. 9241), phospho-IKK α / β (catalog no. 2697) were purchased from Cell Signaling Technology. Normal mouse IgG, rabbit IgG, and antibodies against actin and vinculin were purchased from Sigma. All cells were cultured in Dulbecco's modified Eagle's medium/F12 medium supplemented with 10% fetal bovine serum. HC11 cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 10 ng/ml epidermal growth factor, and 5 μ g/ml insulin.

Quantitative Real-time PCR Analysis—For quantitative real-time PCR (q-PCR), total RNA was extracted using TRIzol reagent (Invitrogen), and first-strand cDNA synthesis was carried out with SuperScript II reverse transcriptase (Invitrogen) using 2 μ g of total RNA and poly(dT) primer. cDNA from macrophages was synthesized using the FastLane Cell cDNA synthesis kit (Qiagen, Valencia, CA). q-PCR was performed with the gene-specific primers listed in [supplemental Table 1](#). q-PCR was performed using a 7900HT sequence detection system (Applied Biosystems, Foster City, CA). The levels of mRNA of all the genes were normalized to those of β -actin mRNA.

Reporter Assays—*Mta1*-luc, interleukin (IL)-1 β -luc, tumor necrosis factor (TNF)- α , and MIP2-luc assays were performed according to the manufacturer's instructions (Promega, Madison, WI), and the results were standardized against the β -galactosidase activity, an internal control. Some assays were performed in the presence of control siRNA or MTA1 siRNA as described previously (20).

Cloning of Murine *Mta1* Promoter and Site-directed Mutagenesis—Full-length murine *Mta1* promoter and its deletion constructs were generated by PCR using mouse genomic DNA and cloned into PGL3 luciferase reporter vector (Promega) using the Infusion 2.0 Dry-Down PCR cloning kit (Clontech, Mountain View, CA). The primers used are listed in [supplemental Table 2](#). The sequence of the constructs was verified by comparing construct sequence with that in the mouse genome database. Mutations in the NF- κ B consensus sequence of murine *Mta1* promoter were created by using the site-directed mutagenesis kit (Stratagene, Cedar Creek, TX) using the primers listed in [supplemental Table 2](#).

Generation of *MTA1*^{-/-} Mice—To generate mice deficient of MTA1, a targeting vector was designed to delete exon 2 by flanking it with LoxP-splicing sites. The targeted construct was introduced into the PC3 embryonic stem cell line, which has a protamine promoter-driven *Cre* transgene, to make clones. Individual clones were injected into C57B6 blastocysts,

male chimera mice were bred with C57B6 female mice, and germ line transmission was confirmed by Southern blotting and PCR assay. The status of the mRNA transcripts from the *Mta1* locus was verified using a pair of primers flanking exon 2. Both lines were kept on a C57B6 and 129Sv mixed background.

Isolation of Peritoneal Macrophage—After LPS treatment, peritoneal lavage was done with 10 ml of sterile ice-cold phosphate-buffered saline, and the peritoneal lavage fluid was collected. The cells were washed and resuspended in Dulbecco's modified Eagle's medium/F12 medium supplemented with 10% fetal bovine serum, cultured overnight, and then washed to remove nonadherent cells.

Immunoprecipitation and Immunoblot Analysis—Cell lysates were prepared in radioimmune precipitation assay lysis buffer, and Western blot analysis was performed as described previously (20). For immunoprecipitation assays, 1 mg of cell lysates was incubated with MTA1 or pol II or HDAC2 or p65 antibody and agarose beads with constant rotation followed by extensive washing (20 mM HEPES (pH 7.6, 150 mM KCl, 1 mM dithiothreitol, 0.1% Nonidet P-40, and 8% glycerol). The immunoprecipitated proteins were resolved on SDS-PAGE, transferred to nitrocellulose membranes, and analyzed by Western blotting using antibodies for NF- κ B signaling components.

siRNA Transfection—siRNA against MTA1 and negative control siRNA were purchased from Dharmacon (Lafayette, CO). Raw cells were seeded at 40% density the day before transfection in 6-well plates, and siRNA transfections were performed with oligofectamine reagent (Invitrogen) according to the manufacturer's instructions. Cells were harvested 48 h after transfection, and cell lysates were prepared.

Chromatin Immunoprecipitation (ChIP)—ChIP assays were performed as described previously (20). Briefly, cells were cross-linked with formaldehyde (1% final concentration) and sonicated on ice to fragment the chromatin into an average length of 500 bp to 1 kb. The lysates were diluted using chromatin-dilution buffer. MTA1 or pol II or p65 or mouse IgG or HDAC2 antibodies were used to immunoprecipitate the respective antigens at 4 °C overnight. Protein A-Sepharose beads saturated with bovine serum albumin, and single-strand DNA were added to the lysate to isolate the antibody-bound complexes. The beads were washed to remove nonspecific binding, and the antibody-bound chromatin was eluted. The eluate was "de-cross-linked" by heating at 65 °C for 6 h. RNase was added during this step to digest the RNA contaminants. Samples were treated with proteinase K for 1 h at 45 °C to digest the proteins pulled down by immunoprecipitation, and, finally, the DNA was extracted using the phenol chloroform method. For the double ChIP experiment, an initial ChIP assay was done with either MTA1 or p65 antibody to immunoprecipitate MTA1-bound chromatin, which was eluted from the protein A-Sepharose beads and subjected to a second ChIP assay with either pol II or anti-HDAC2 antibody. With the DNA isolated at the end of the ChIP analysis, PCR was performed using the primers mentioned in [supplemental Table 3](#).

EMSA—Nuclear extracts were prepared using a Nonidet P-40 lysis method (21). EMSA for NF- κ B DNA binding was performed using the annealed and [γ -³²P]ATP end-labeled NF- κ B consensus oligonucleotides in a 20- μ l reaction mix-

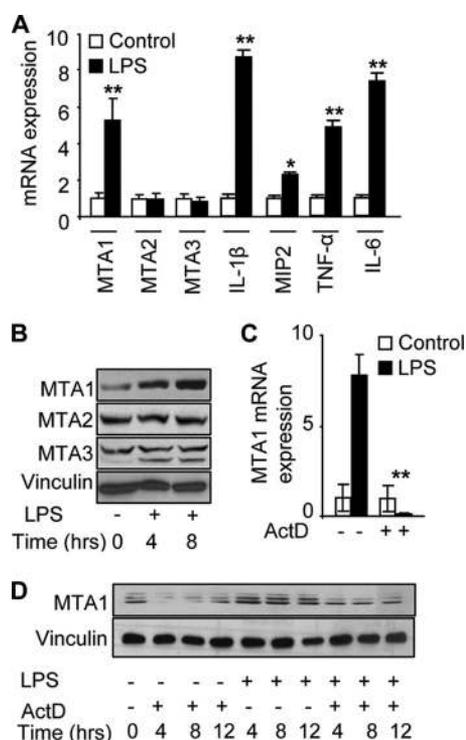


FIGURE 1. MTA1 is target of inflammation. A, q-PCR analysis of MTA1, MTA2, MTA3, IL-1 β , MIP2, TNF- α , and IL-6 mRNA in murine peritoneal macrophages treated with LPS for 2 h. B, Western blot analysis of MTA1, MTA2, and MTA3 protein in Raw cells treated with LPS at different time points (in h). C, q-PCR analysis of MTA1 mRNA in Raw cells treated with LPS with or without actinomycin-D (ActD) for 4 h. D, Western blot analysis of MTA1 protein in Raw cells treated with LPS with or without ActD at different time points. *, $p < 0.05$; **, $p < 0.001$.

ture for 15 min at 20 °C. Samples were run on a nondenaturing 5% polyacrylamide gel and imaged by autoradiography. Specific competitions were performed by adding a 100-molar excess of competitor to the incubation mixture, and supershift EMSAs were performed by adding 1.5 μ l of the NF- κ B p65 (286-H, Santa Cruz Biotechnology). The oligonucleotides used in EMSA were mentioned in supplemental Table 4.

Determination of HDAC2 S-Nitrosylation Was Done by Biotin Switch Method—S-nitrosylation of HDAC2 was detected by using the standard biotin-switch method. The assay was performed using Cayman Chemical's S-nitrosylation detection kit. Briefly, biotinylated proteins were pulled down with streptavidin-agarose beads, and immunoblot analysis was performed to detect the amount of total HDAC2.

Statistical Analysis and Reproducibility—The results are given as the means \pm S.E. Statistical analysis of the data were performed by using Student's *t* test.

RESULTS AND DISCUSSION

MTA1 Is an Inflammation-inducible Gene—During an investigation involving treatment of primary murine peritoneal macrophages with *Escherichia coli* LPS, we discovered unexpectedly that LPS stimulation of macrophages results in a substantial induction of MTA1 mRNA but not MTA2 mRNA or MTA3 mRNA as measured by q-PCR (Fig. 1A). As expected, increased expressions of IL-1 β , MIP2, IL-6, and TNF- α , the

primary targets of LPS, were observed (Fig. 1A). The results suggest that MTA1 may be an inflammation-inducible gene. Therefore, to test this hypothesis, we turned to LPS stimulation of Raw264.7 cell line (Raw cells), a widely used murine macrophage cell line, as a model system. We found that LPS stimulation led to increased expression of MTA1 protein (Fig. 1B) and mRNA as assessed by q-PCR (Fig. 1C). In contrast, LPS stimulation had no effect on the expression of MTA2 or MTA3 protein (Fig. 1B). Actinomycin-D, a transcriptional inhibitor, effectively blocked both LPS-inducible expression of MTA1 mRNA and protein (Fig. 1, C and D). These findings suggested that MTA1 may be a target of inflammation.

MTA1 Is an NF- κ B-responsive Gene—To directly test the notion that MTA1 is an inflammation-inducible gene, we next cloned the putative murine *Mta1* promoter from mouse genomic DNA and generated a series of overlapping promoter fragments into a TATA-less, pGL3-luciferase (pGL3-luc) reporter system (supplemental Fig. 1A). We examined the inducibility of the *Mta1* promoter deletion constructs by TNF- α , the primary target of LPS (supplemental Fig. 1B). These experiments led to identification of the *Mta1* minimal promoter, which consisted of -2872 to -5200. A close examination of the *Mta1* minimal promoter revealed the presence of five NF- κ B consensus motifs: GGAAAGCCAG (-4761 to -4770), GAAAACCCCA (-3926 to -3935), TTCCCAGAGG (-3967 to -3996), AATTCCTCTG (-3986 to -3995), and AAGAGGGGTC (-2893 to -2902) (supplemental Fig. 1C), suggesting that in principle, MTA1 can be induced by inflammatory signals. Indeed, LPS (and TNF- α) rapidly stimulated MTA1 transcription from the luc reporter system in Raw and in murine HC11 mammary epithelial cells (Fig. 2A). As the goal of this study was to integrate the role of MTA1 during inducible inflammation, we used LPS as a physiologically relevant activator in the subsequent studies.

To understand the mechanism of LPS regulation of MTA1 expression, we found that blocking the NF- κ B pathway by either pharmacologic inhibitor parthenolide or I κ B α -S12 (22), a specific dominant-negative mutant of I κ B, effectively inhibited LPS-mediated stimulation of *Mta1* promoter activity in Raw cells (supplemental Fig. 2). As expected, LPS-mediated stimulation of MTA1 mRNA expression was also inhibited by parthenolide (Fig. 2B).

To gain a deeper insight into LPS stimulation of MTA1 transcription, we next used ChIP to examine the potential recruitment of p65 onto the *Mta1* promoter. Although the *Mta1* promoter contains five putative NF- κ B consensus motifs (supplemental Fig. 1C), LPS stimulation resulted in enhanced recruitment of p65 only to region 1 (-4570 to -4889) (Fig. 2C) and not to regions 2 (-3814 to -4152) and 3 (-2874 to -3207) (Fig. 2C). In addition, we also found recruitment of pol II, a marker of stimulated chromatin, to this region (Fig. 2C). Interestingly, we also found a distinct derecruitment of HDAC2 from region 1 of the *Mta1* promoter upon LPS treatment (Fig. 2C). Similarly, p65-pol II complex was recruited only to region 1 of the *Mta1* promoter as revealed by sequential double ChIP studies (Fig. 2D).

To demonstrate the functionality of the responsible NF- κ B motif in the *Mta1* promoter, we next mutated the NF- κ B

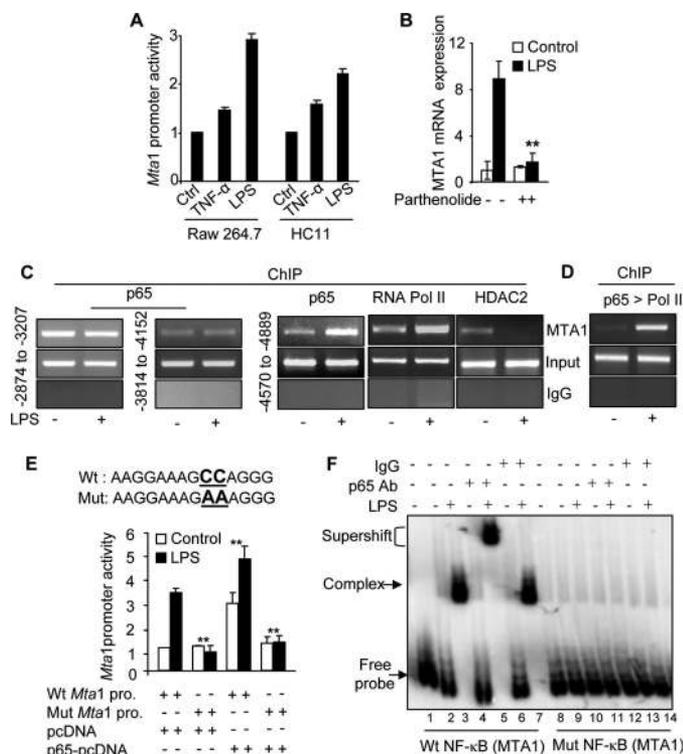


FIGURE 2. MTA1 is an NF- κ B-responsive gene. *A*, *Mta1* promoter activity in indicated cells treated with LPS or TNF- α for 4 h. *B*, q-PCR analysis of MTA1 in Raw cells treated with LPS in the presence of parthenolide (5 μ M) for 4 h. All data are shown as mean \pm S.D., $n = 3$, * $p < 0.05$, ** $p < 0.001$. *C*, recruitment of p65 or pol II or HDAC2 to 320 bp (–4570 to –4889) *Mta1* chromatin by ChIP assay in Raw cells treated with LPS for 1 h. *D*, recruitment of p65 followed by pol II complex to 320 bp (–4570 to –4889) *Mta1* chromatin by sequential double ChIP assay in Raw cells treated with LPS for 1 h. *E*, promoter activities of *Mta1* promoter (Wt) and *Mta1* promoter harbored indicated mutation points (Mut) after cotransfecting either with pcDNA or p65 expression vector in Raw cells treated with LPS. Data are shown as mean \pm S.D., $n = 3$, ** $p < 0.001$. *F*, EMSA analysis of p65 binding to the mouse *Mta1* promoter using the oligonucleotide encompassing the wild-type and mutant NF- κ B consensus sequence in Raw cells treated with LPS for 1 h. *pro.*, promoter, *Ab*, antibody; *Ctrl*, control.

motif in region 1 of the *Mta1* promoter-luc reporter and showed that both LPS and p65 fail to stimulate the *Mta1* promoter-luc activity containing a mutant NF- κ B consensus motif (Fig. 2*E*). To demonstrate a potential direct binding of p65 to the mouse *Mta1* promoter, we next performed EMSA using a 24-bp oligonucleotide encompassing the wild-type and mutant NF- κ B consensus sequence of *Mta1* promoter and nuclear extracts from Raw cells with or without LPS stimulation. As expected from the preceding results, LPS stimulation of Raw cells promoted higher levels of p65-DNA complex formation than those in the unstimulated Raw cells. The specificity of the noted complex was verified by supershift experiments using anti-p65 or control IgG (Fig. 2*F*, lanes 4–7) and by using MTA1 DNA probe with a defective NF- κ B consensus sequence that did not interact with the cellular p65 (lanes 8–14). Together, these findings suggested that LPS stimulates *MTA1* transcription via the NF- κ B pathway and that *MTA1* is an NF- κ B-responsive gene.

MTA1 Is a Modifier of the LPS-inducible Genes in Stimulated Macrophage—We next examined the possibility that MTA1 is a new modifier of NF- κ B signaling in LPS-stimulated macrophage. We found that selective siRNA-mediated knockdown of

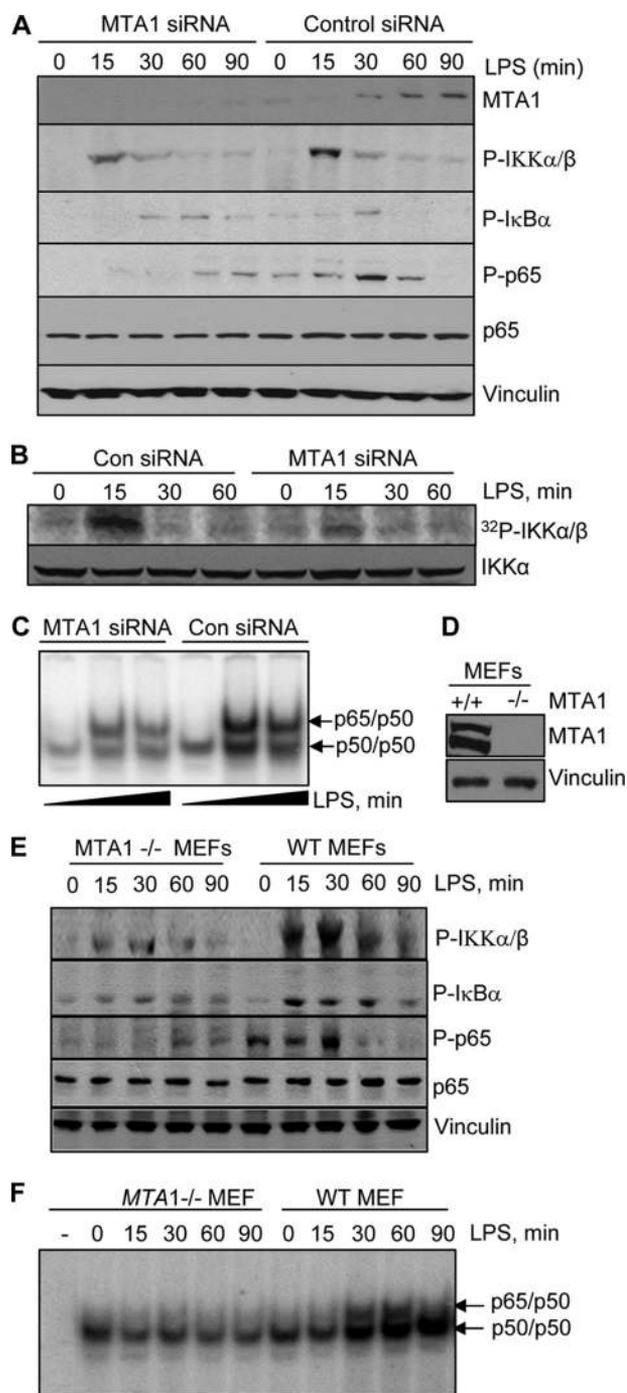


FIGURE 3. MTA1 is a new modifier of NF- κ B signaling in stimulated macrophage. *A*, effect of selective knockdown of MTA1 on the activation status of the NF- κ B signaling components in LPS-stimulated Raw cells by Western blot analysis. *B*, kinase assay for the p-IKK α in LPS-stimulated Raw cells with or without MTA1 knockdown. *C*, EMSA analysis of the p65-DNA complex in LPS (dark triangles: 0, 30, and 60 min) stimulated Raw cells with or without MTA1 knockdown. *D*, Western blot analysis for MTA1 in *MTA1*^{+/+} and *MTA1*^{-/-} MEFs. *E*, effect of LPS on the activation status of NF- κ B signaling components in wild-type (WT) and *MTA1*^{-/-} MEFs by Western blot analysis. *F*, EMSA analysis of the p65-DNA complex in LPS-stimulated wild-type and *MTA1*^{-/-} MEFs. *Con.*, control.

MTA1 in Raw cells impaired LPS-induced early activation of phospho-IKK α (Fig. 3*A*), IKK α kinase activity (Fig. 3*B*), and also delayed I κ B α phosphorylation and p65 phosphorylation as compared with those observed in cells transfected with the con-

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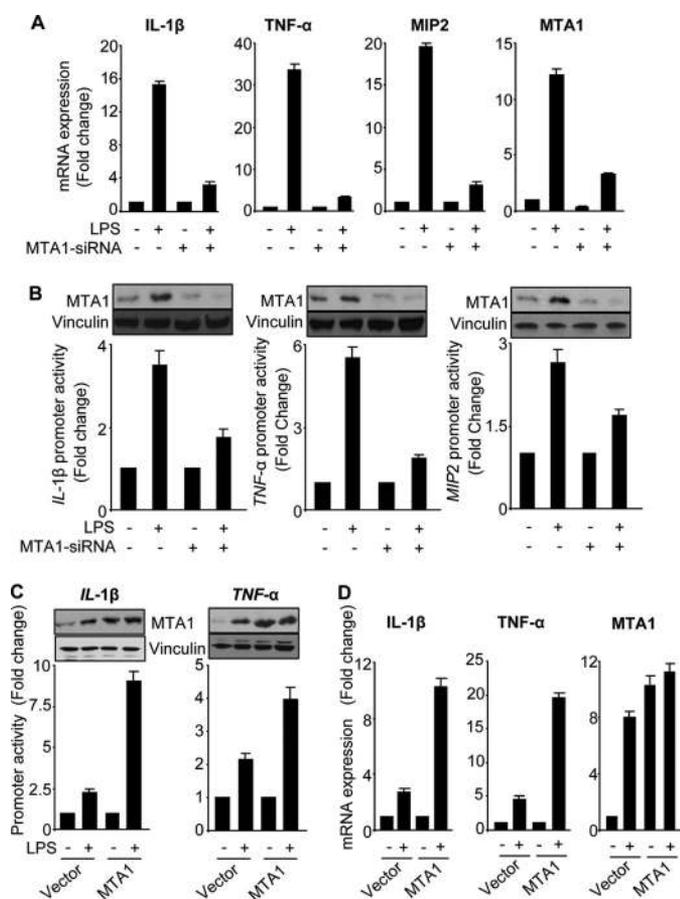


FIGURE 4. MTA1 stimulates NF- κ B responsive genes in activated macrophages. *A*, q-PCR analysis of IL-1 β , TNF- α , and MIP2 mRNAs in LPS-stimulated Raw cells with or without MTA1 knockdown. Results were presented in fold change. *B*, promoter luc activity of IL-1 β , TNF- α , and MIP2 in LPS-stimulated Raw cells with or without MTA1 knockdown. Results were presented in fold change. *C*, promoter activity of IL-1 β and TNF- α in LPS-stimulated Raw cells after cotransfecting with vector pcDNA or MTA1. Results were in fold change. *D*, q-PCR analysis of IL-1 β and TNF- α mRNA in LPS-stimulated Raw cells after cotransfecting with vector pcDNA or MTA1. Results were presented in fold change. All data are shown as mean \pm S.D., $n = 3$.

control siRNA (Fig. 3A). Consistent with these observations, there also was reduced binding of activated p65 to the NF- κ B consensus DNA element in LPS-stimulated MTA1 knockdown Raw cells as compared with control unstimulated cells (Fig. 3C). Similarly, we found an overall reduction in the activation of NF- κ B signaling components (Fig. 3E) and the DNA-binding ability of p65 (Fig. 3F) in LPS-stimulated MTA1^{-/-} mouse embryonic fibroblasts (MEFs) (Fig. 3D) as compared with those of LPS-stimulated wild-type MEFs. These findings suggested that MTA1 status is an important modifier of NF- κ B signaling in LPS-stimulated macrophages.

We next examined whether MTA1 exerts a modifying function on NF- κ B target genes in stimulated cells. We found that selective siRNA-mediated depletion of the endogenous MTA1 in Raw cells led to a significant fold reduction in LPS-induced expression of IL-1 β , TNF- α , and MIP2 as compared with those of siRNA control cells (Fig. 4A and supplemental Fig. 3A). Consistent with these findings, MTA1 knockdown in Raw cells reduced the efficacy of LPS-induced transcription of IL-1 β , TNF- α , and MIP2 from the basal level as compared with those in control siRNA-transfected cells, evaluated by the respective

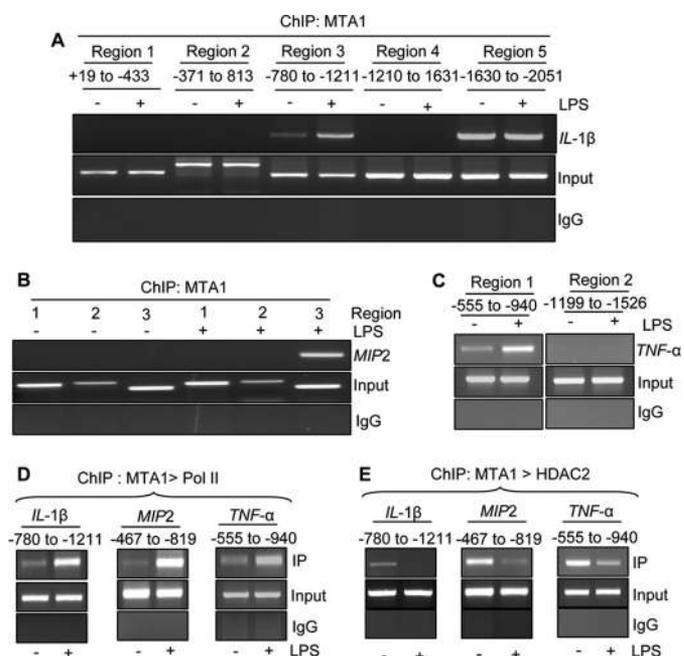


FIGURE 5. Comodifying effect of MTA1 on NF- κ B target genes. *A–C*, ChIP analysis of MTA1 recruitment to IL-1 β , MIP2, and TNF- α chromatin in Raw264.7 cells. *D*, ChIP analysis of MTA1 followed by pol II complex recruitment to IL-1 β (–780 to –1211), MIP2 (–467 to –819), and TNF- α (–555 to –940) chromatin in Raw cells treated with LPS for 1 h. *E*, ChIP analysis of MTA1 followed by HDAC2 complex recruitment to IL-1 β (–780 to –1211), MIP2 (–467 to –819), and TNF- α (–555 to –940) chromatin in Raw cells treated with LPS for 1 h. *Input* and *IgG* are the chIP controls.

promoter luc reporters (Fig. 4B and supplemental Fig. 3B). These findings implied that MTA1 may be important in achieving optimum induction of NF- κ B target genes by LPS, suggesting that MTA1 may play a modifying role in the responsiveness of macrophages to LPS. To directly evaluate this possibility, we next examined the effect of ectopic MTA1 expression on LPS-mediated stimulation of IL-1 β and TNF- α transcription using the respective promoter luc reporters (Fig. 4C). MTA1 expression in Raw cells potentiated the effect of LPS on NF- κ B target genes (IL-1 β and TNF- α) transcription as compared with what was observed in control vector-transfected cells. Similarly, MTA1 overexpression was accompanied by increased levels of LPS-mediated stimulation of IL-1 β and TNF- α expression in Raw cells as compared with vector-transfected control cells (Fig. 4D).

To further examine the potential comodifying role of MTA1 in LPS-mediated stimulation of the NF- κ B target genes, we next examined whether LPS stimulation of macrophages also is accompanied by the recruitment of MTA1-pol II complex to NF- κ B target gene promoters. Using a ChIP-based promoter walk, we first established that indeed, MTA1 is recruited to region 3 (–780 to –1211) of the IL-1 β promoter, region 3 (–467 to –819) of the MIP2 promoter, and region 1 (–555 to –940) of the TNF- α promoter (Fig. 5, A–C). We observed that in Raw cells, LPS stimulation promoted recruitment of the MTA1-pol II complex to the IL-1 β , TNF- α , and MIP2 promoters (Fig. 5D). Together, these results suggested that MTA1 behaves as a modifier of the LPS-inducible genes in the activated macrophages.

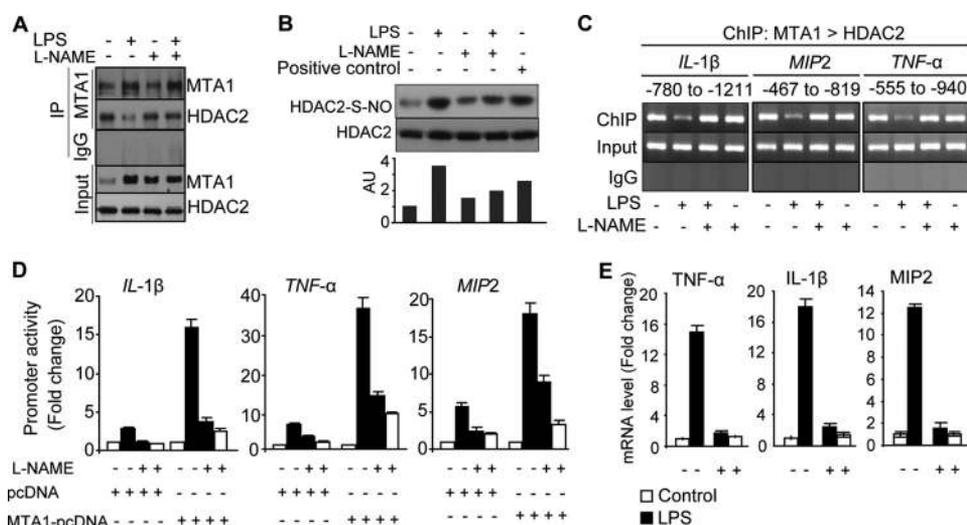


FIGURE 6. HDAC2 nitrosylation results in loss of corepressor activity of MTA1-NuRD complex. *A*, Western blot analysis of HDAC2 and MTA1 after IP with MTA1 in Raw cells treated with LPS for 4 h in the presence and absence of L-NAME. *B*, S-nitrosylation of HDAC2 was assessed using the biotin switch assay after treating the Raw 264.7 cells with LPS and sodium nitroprusside (positive control) for 4 h. *C*, ChIP analysis of MTA1 followed by HDAC2 complex recruitment to IL-1 β (-780 to -1211), MIP2 (-467 to -819), and TNF- α (-555 to -940) chromatin in Raw cells treated with LPS for 1 h in the presence and absence of L-NAME. *D*, promoter activities of IL-1 β , TNF- α , and MIP2 in LPS-stimulated Raw cells after cotransfecting with vector pcDNA or MTA1 in the presence and absence of L-NAME. Results were presented in fold change. *E*, q-PCR analysis of TNF- α , IL-1 β , and MIP2 mRNA in LPS-stimulated Raw cells in the presence and absence of L-NAME. All data are shown as mean \pm S.D. *Au*, arbitrary units; *S-NO*, S-nitrosylation; *pcDNA*, pcDNA vector; *Input* as loading control.

Revelation of Corepressor Activity of MTA1 on a Subset of NF- κ B-regulated Genes in Resting Cells—During the course of the above studies, we consistently noticed that MTA1 knockdown in macrophages was accompanied by a distinct inhibition of the fold change in the levels of IL-1 β , TNF- α , and MIP2 upon LPS treatment compared with those in LPS-stimulated wild-type Raw cells (Fig. 4A). Most notably, in MTA1 knockdown cells, the mRNA basal levels of these NF- κ B-responsive genes and their basal promoter activity were elevated (supplemental Fig. 3). These findings raised an interesting possibility that MTA1 might act as a corepressor of NF- κ B target genes under basal, unstimulated conditions. Because MTA1 is part of the HDAC-NuRD corepressor complex, this hypothesis also was supported by our finding that LPS stimulation of macrophages was accompanied by derecruitment of the MTA1-HDAC2 corepressor complexes from the LPS-inducible NF- κ B target gene chromatin: IL-1 β , TNF- α , and MIP2 genes (Fig. 5E). Together, these results suggested that 1) MTA1 may repress the basal transcription of a subset of LPS-regulated target genes owing to a constitutive recruitment of the MTA1-HDAC2 complexes to the target promoter chromatin under unstimulated condition and 2) MTA1 acts as a comodifier of a subset of NF- κ B target genes in stimulated macrophages, probably owing to the release of HDAC2 from its target chromatin, probably, allowing the recruitment of pol II coactivator complexes to the target promoter chromatin. However, the precise nature of LPS-responsive components and associated protein modifications, which, in turn, might contribute to the noted recruitment of pol II coactivator complexes remains to be further investigated.

S-Nitrosylation of HDAC2 Abolishes Corepressor Activity of the MTA1-NuRD Complex—We next explored the notion that differential HDAC2 interaction with the NF- κ B target gene chromatin during basal and LPS-inducible conditions

may influence the status of MTA1 being a corepressor or loss of corepressor activity. We examined the effect of LPS stimulation on the relative interaction of MTA1 with HDAC2. We found that LPS stimulation of Raw cells led to a decreased interaction between MTA1 and HDAC2 in spite of increased expression of MTA1 by LPS (Fig. 6A). While this study was in progress, Nott *et al.* have shown that growth factor stimulation of nitric oxide (NO) production, via inducible nitric-oxide synthase, induces S-nitrosylation of nuclear HDAC2. Nitrosylated HDAC2, as a result, is released from the chromatin, leading to the loss of corepressor function and the stimulation of target gene transcription (23). In this context, because inducible nitric oxide synthase also is an NF- κ B target gene (24), we discovered that LPS also stimulates S-nitrosylation of

HDAC2 as detected by biotin switch assay (Fig. 6B, compare lane 2 with 1) and that nitrosylated HDAC2 modification could be inhibited by the inclusion of a pharmacological inducible nitric-oxide synthase inhibitor L-NAME (Fig. 6B, compare lane 4 with 2). L-NAME also reversed the loss of interaction between HDAC2 and MTA1 in LPS-treated Raw cells (Fig. 6A). Consistent with the potential implication of S-nitrosylation of HDAC2 on the corepressor activity of the MTA1-HDAC2 complex, we found that LPS-mediated derecruitment of the MTA1-HDAC2 corepressor complex from the representative NF- κ B target gene promoters such as IL-1 β , TNF- α , and MIP2 promoter chromatin (Fig. 6C) could be effectively reversed by inducible nitric oxide synthase inhibitor L-NAME. To implicate these molecular changes with the functionality of the MTA1-HDAC2 corepressor complex, we showed that indeed, treatment of Raw cells with L-NAME, which prevented the release of MTA1-HDAC2 corepressor complex from IL-1 β , TNF- α , and MIP2 promoters, also resulted in the failure in stimulating IL-1 β , TNF- α , and MIP2 promoter activities by LPS (Fig. 6D) as well as expression of these LPS-inducible genes (Fig. 6E). Together, these findings suggest that the modulation of S-nitrosylation status of HDAC2 and its differential interaction with MTA1 in resting versus LPS-stimulated macrophages provides an explanation for the noted loss of corepressor behavior of MTA1 in activated macrophages.

MTA1 Modulation of Host Inflammatory and Septic Shock Responses—To confirm the possible differential effects of MTA1 knockdown under basal and LPS-stimulated conditions in a whole-animal setting, we used MTA1^{-/-} mice, which were generated recently in our laboratory (25). We examined the levels of a subset of NF- κ B target genes in cultured peritoneal macrophages from wild-type and MTA1^{-/-} mice treated with *E. coli* LPS for 4 h. We found that MTA1

Regulation of NF- κ B Circuitry Controls Homeostasis

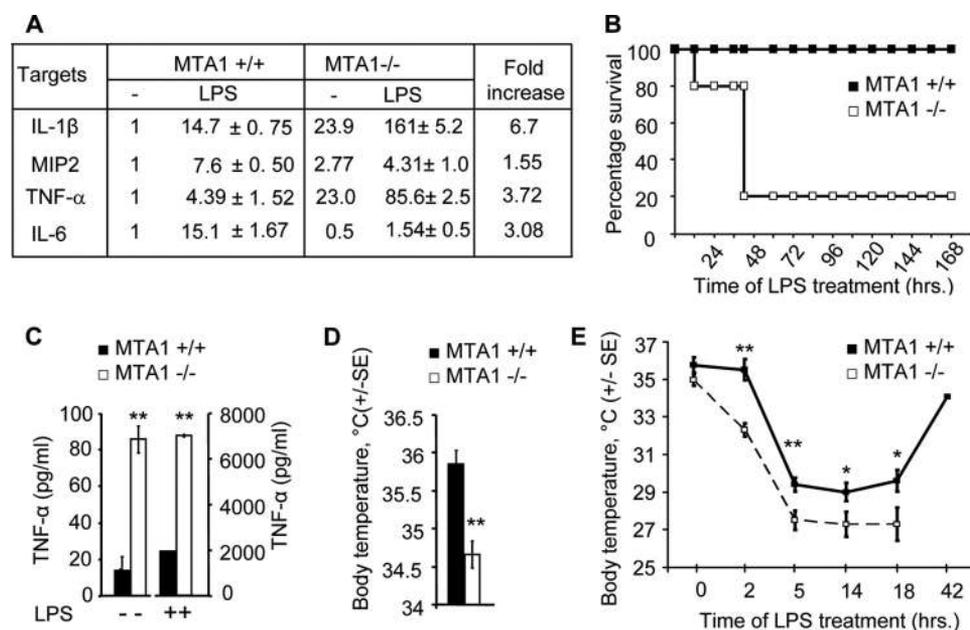


FIGURE 7. MTA1 modulation of host inflammatory and septic shock responses. A, q-PCR analysis of indicated genes (data presented are in fold change) in macrophages isolated from either wild-type ($MTA1^{+/+}$) or $MTA1$ knock-out ($MTA1^{-/-}$) mice treated with LPS for 2 h. B, survival rates of mice in h after intraperitoneal injection of LPS (10 mg/kg) ($n = 5$ each group). C, estimation of TNF- α levels in circulation of wild-type or $MTA1^{-/-}$ mice before and after 2 h of LPS treatment by enzyme-linked immunosorbent assay. D, $MTA1^{-/-}$ mice showing significant difference in the base level body temperature as compared with those of the wild-type mice ($n = 19$ /group). E, effect of LPS on the body temperature of wild-type and $MTA1^{-/-}$ mice. All of the $MTA1^{-/-}$ mice died after 18 h of LPS treatment. Data are shown as mean \pm S.D., $n = 3$, *, $p < 0.05$, **, $p < 0.001$.

ones, fold change in cytokines expression between stimulated and basal in $MTA1^{-/-}$ macrophages were significantly lower than those of wild-type (Fig. 7A). Although these results apparently are paradoxical, they nevertheless are consistent with the findings in Raw cells and support the notion that MTA1 acts as a corepressor of a subset of the NF- κ B target genes under basal conditions but acts as a modifier of the some of these genes in LPS-stimulated macrophages, suggesting a contribution of LPS-induced signals in the noted reversal of the basal corepressor activity.

To understand the impact of MTA1 deficiency on inflammatory responses, we examined the comparative susceptibility of $MTA1^{-/-}$ mice and wild-type mice to septic shock induced by *E. coli* LPS. Consistent with the findings of dramatic inflammatory response and increased expression of proinflammatory cytokines that mediates innate immune response, we observed a high rate of pronounced death of $MTA1^{-/-}$ mice within the first 40 h, whereas the wild-type mice survived during this period (Fig. 7B), presumably due to extremely high levels of circulating TNF- α , at least as one of the mechanisms (Fig. 7C), revealing a maintenance role of MTA1 in inflammatory response. As expected given the increased baseline expression of cytokines in macrophages from $MTA1^{-/-}$ mice (Fig. 7A), the normal body temperature of $MTA1^{-/-}$ mice was $\sim 1^\circ\text{C}$ lower than the normal body temperature of the wild-type mice (Fig. 7D). Interestingly, LPS-induced septic shock triggered a further severe and lasting hypothermia in the $MTA1^{-/-}$ mice (body temperatures

dropped to 27.3°C), whereas in the wild-type mice, body temperature dropped to only 29°C . However, the temperature returned to near the starting normal body temperature of 34°C after LPS-induced decrease in body temperature in the wild-type animals (Fig. 7E). These findings suggested that MTA1 levels may provide an inherent protection in mice from deregulated host inflammatory response under conditions of septic shock and bacterial infection.

In summary, the results presented here have identified a critical role of MTA1, both as a target and as a modifier of the

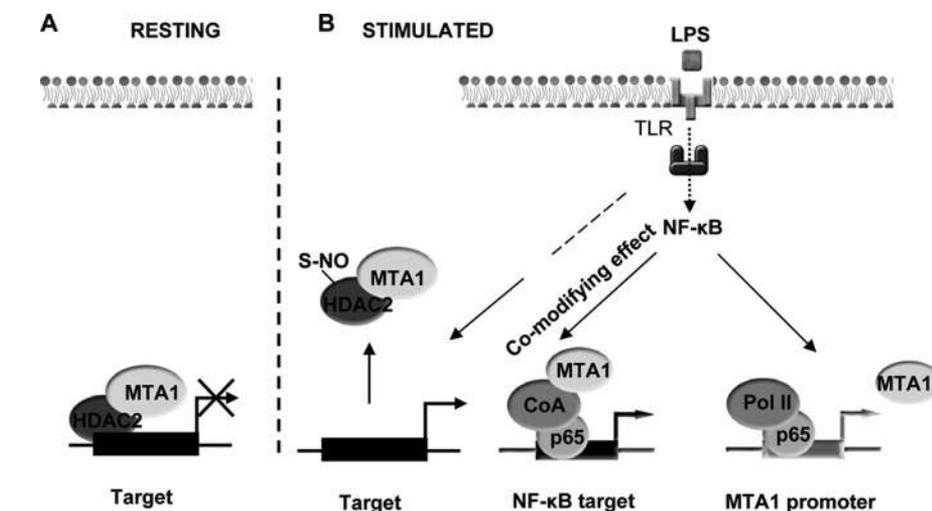


FIGURE 8. Working Model. A, repression of a subset of proinflammatory cytokines by the MTA1-HDAC2 corepressor complex in the resting macrophages. B, transcriptional stimulation of MTA1 expression by the NF- κ B pathway in LPS-stimulated macrophages. LPS enhances S-nitrosylation of HDAC2, leading to its dissociation from the MTA1-HDAC2 complex as well as from the target promoter; this, in turn, facilitates co-modifying role of MTA1 on the expression of a subset of NF- κ B genes. TLR, Toll-like receptor, S-NO, S-nitrosylation.

NF- κ B pathway, in conferring an optimal, sufficient inflammatory response against bacterial infection. As a mechanism of MTA1 regulation of a subset of LPS-inducible proinflammatory cytokines, the MTA1-HDAC2 corepressor complex differentially interacts with and remodels the target gene chromatin under basal and LPS-stimulated conditions (Fig. 8). As a result, the noted regulation of MTA1 by inflammation and the impact of MTA1 on NF- κ B signaling and NF- κ B target gene chromatin remodeling most likely are involved in the overall outcome of NF- κ B signaling in macrophages. In conclusion, the findings presented here have identified a new regulatory layer of NF- κ B signaling during inflammation and indicate that MTA1 represents as a player in and modifier to the NF- κ B signaling network to, having a role in maintaining the homeostasis of inflammatory responses.

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