1-1-2008

A novel CCCH-zinc finger protein family regulates proinflammatory activation of macrophages

Jian Liang
University of Central Florida

Jing Wang
University of Central Florida

Asim Azfer
University of Central Florida

Wenjun Song
University of Central Florida

Gail Tromp
University of Central Florida

See next page for additional authors

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 283, NO. 10, pp. 6337–6346, March 7, 2008

A Novel CCCH-Zinc Finger Protein Family Regulates Proinflammatory Activation of Macrophages*

Received for publication, September 19, 2007, and in revised form, December 24, 2007 Published, JBC Papers in Press, January 4, 2008, DOI 10.1074/jbc.M707861200

Jian Liang, Jing Wang, Asim Azfer, Wenjun Song, Gail Tromp, Pappachan E. Kolattukudy, and Mingui Fu[1]

From the Biomolecular Science Center, College of Medicine, University of Central Florida, Orlando, Florida 32816

Activated macrophages play an important role in many inflammatory diseases. However, the molecular mechanisms controlling macrophage activation are not completely understood. Here we report that a novel CCCH-zinc finger protein family, MCPIP1, 2, 3, and 4, encoded by four genes, *Zc3h12a*, *Zc3h12b*, *Zc3h12c*, and *Zc3h12d*, respectively, regulates macrophage activation. Northern blot analysis revealed that the expression of MCPIP1 and MCPIP3 was highly induced in macrophages in response to treatment with lipopolysaccharide (LPS). Although not affecting cell surface marker expression and phagocytotic function, overexpression of MCPIP1 significantly blunted LPS-induced inflammatory cytokine and NO_2^- production as well as their gene expression. Conversely, short interfering RNA-mediated reduction in MCPIP1 augmented LPS-induced inflammatory gene expression. Further studies demonstrated that MCPIP1 did not directly affect the mRNA stability of tumor necrosis factor α and monocyte chemoattractant protein 1 (MCP-1) but strongly inhibited LPS-induced tumor necrosis factor α and inducible nitric-oxide synthase promoter activation. Moreover, we found that forced expression of MCPIP1 significantly inhibited LPS-induced nuclear factor-κB activation. These results identify MCP-induced proteins, a novel CCCH-zinc finger protein family, as negative regulators in macrophage activation and may implicate them in host immunity and inflammatory diseases.

Macrophages play an important role in many inflammatory disease states, including atherosclerosis, rheumatoid arthritis, emphysema, pulmonary fibrosis, and chronic pancreatitis. In these disease settings activated macrophages elaborate a large array of cytokines, growth factors, and proteolytic enzymes that are critical for tissue damage and repair (1). Numerous extracellular inducers of macrophage activation have been identified, among the most studied of which are lipopolysaccharide (LPS)[2] and interferon γ (IFNγ) (2). LPS is a structurally heterogenous material contained within the cell wall of Gram-negative bacteria and is recognized by animals as a molecule correlating to infection. It binds to Toll-like receptor 4 (TLR4), triggering multiple signaling cascades including those mediated through the transcription factor NF-κB and the Janus N-terminal kinase and p38 kinase pathways (3).

Much attention has focused on the pro-inflammatory signaling in activated macrophages but little is known about the mechanisms that negatively control inflammation. Understanding the molecular mechanisms involved in the inflammatory processes in macrophages is essential to the development of novel drug therapies against inflammatory diseases, including atherosclerosis.

Previously, we identified a novel CCCH-zinc finger-containing protein that was significantly induced by MCP-1 in human peripheral blood monocytes and thus designated as MCP-induced protein (MCPIP) (4). By searching the similar sequences in GenBank™, we found that four members belong to this protein family, designated as MCPIP1, 2, 3, and 4 and encoded by four genes, *Zc3h12a*, *Zc3h12b*, *Zc3h12c*, and *Zc3h12d*, respectively. The obvious feature of this protein family is that they all contain a single CCCH-type zinc finger and their function remains unknown.

Zinc finger proteins comprise a large superfamily. It is estimated that 1% of all mammalian genes encode zinc fingers. There are at least 14 different types of fingers, categorized by the nature and spacing of their zinc-chelating residues. Most zinc fingers are CCHH or CCCC type (referred to as classical zinc fingers). CCCH-type zinc finger-containing proteins are very unusual in mammalian genomes and represent ~0.8% of all zinc finger-containing proteins (5, 6). A well studied CCCH-zinc finger protein family is the tristetraprolin (TTP, also known as Zfp36) family, which also contains four members, TTP, Zfp36l1, Zfp36l2, and Zfp36l3. This protein family contains two tandem CCCH-zinc fingers and binds to AU-rich elements in mRNA, leading to the removal of the poly(A) tail from that mRNA and increased rates of mRNA turnover (7–9). ZAP, which is also a CCCH-type zinc finger protein, can bind to viral RNA to cause profound and specific loss of viral mRNA (10). Roquin, another CCCH-zinc finger protein, functions as a ubiquitin ligase and is required to repress autoimmunity (11).

In this study, we found that the members of the MCPIP family were dramatically induced by LPS in macrophages. Overexpression of MCPIP1 significantly attenuated LPS-induced inflammatory cytokine and NO_2^- production and their gene

* This work was supported by UCF Starting Package (to M. F.) and National Institutes of Health Grant HL69458 (to P. E. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] To whom correspondence may be addressed: Biomolecular Science Center, College of Medicine, University of Central Florida, 4000 Central Florida Blvd., Orlando, FL 32816. Tel.: 407-823-1306; Fax: 407-823-0956; E-mail: mfu@mail.ucf.edu.

[2] The abbreviations used are: LPS, lipopolysaccharide; IFNγ, interferon γ; TNFα, tumor necrosis factor α; IL-1β, interleukin 1β; ARE, AU-rich element; FBS, fetal bovine serum; TTP, tristetraprolin; iNOS, inducible nitric-oxide synthase; NF-κB, nuclear factor κB; EGFP, enhanced green fluorescent protein; PBS, phosphate-buffered saline; ac-LDL, acetylated low-density lipoprotein; PPRE, peroxisome proliferator-activated receptor response element.

expression, perhaps by antagonizing NF-κB signaling. These results indicate that they may be novel regulators in macrophage activation.

MATERIALS AND METHODS

Mice—Male C57BL/6 wild-type mice (~6–8 weeks old) were purchased from The Jackson Laboratories (Bar Harbor, ME) and housed in a temperature-controlled environment with 12-h light/dark cycles at the University of Central Florida Transgenic Animal Facility in accordance with the principles of animal care of the National Institutes of Health. These studies were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Central Florida.

Cells—The mouse macrophage cell line RAW264.7 was obtained from American Type Culture Collection (ATCC) and maintained in RPMI 1640 supplemented with 2 mM glutamine, 100 units/ml of penicillin and streptomycin, and 10% fetal bovine serum (FBS) (endotoxin <1 ng/ml) (Sigma). The human acute monocytic leukemia cell line THP-1 was obtained from ATCC and maintained in RPMI 1640 medium with 2 mM L-glutamine, 1.5 g/liter sodium bicarbonate, 4.5 g/liter glucose, 10 mM HEPES, and 1.0 mM sodium pyruvate and supplemented with 0.05 mM 2-mercaptoethanol, 10% FBS. THP-1 cells were stimulated with 100 nM/liter phorbol 12-myristate 13-acetate for 5 days to differentiate into macrophages and then were quiescent for 16–24 h by being cultured in macrophage serum-free medium (Invitrogen).

Mouse bone marrow-derived macrophages were generated from bone marrow stem cells obtained from femurs of male C57BL/6 mice (2–4 months old). After lysis of the red blood cells, 4×10^6 of bone marrow stem cells were inoculated in 6-well plates with complete Dulbecco's modified Eagle's medium culture medium containing 10% FBS, 30% L929 conditional medium, and standard supplements. After 7 days of culture, the fully differentiated and matured bone marrow-derived macrophages were quiescent for 24 h in macrophage serum-free medium and then treated with 1 μg/ml LPS for different time points. Human embryonic kidney 293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 2 mM glutamine, 100 units/ml of penicillin and streptomycin, and 10% FBS.

Plasmids—Human MCPIP1 expression plasmid (pMCPIP1) was generated as described previously (4). An open reading frame of human MCPIP1 was ligated to the N terminus of EGFP within the vector pEGFP-N1 by using EcoRI and BamHI. In the transient transfection experiment, pEGFP-N1 was used as a control. βWt (containing human β-globin gene) and βWt-TNFα (containing β-globin gene and the potent ARE from tumor necrosis factor α mRNA in the 3′-untranslated region) were kindly provided by Dr. Jens Lykke-Andersen (University of Colorado, Boulder) (12). TNFα-Luc plasmid was a gift from Dr. Dmitry Kuprash (Russian Academy of Science) (13). iNOS-Luc and PPRE-TK-Luc were from the laboratory of Dr. Mangelsdorf (UT Southwestern, Dallas, TX). NF-κB-TK-Luc was purchased from Stratagene (La Jolla, CA).

Reagents—The MCPIP1 rabbit polyclonal antibody was prepared against the human recombinant MCPIP1 protein as described previously (4). B7-1 (sc-1632) and actin antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rhodamine-conjugated goat anti-rabbit IgG was from Chemicon International (Temecula, CA). LPS (*Escherichia coli* 026:B6-derived), human recombinant interferon-γ, and actinomycin D were purchased from Sigma. Doxycycline was from Clontech (Mountain View, CA).

Transfection—For functional study, transient transfection into Raw264.7 cells was performed by electroporation following the manufacturer's instruction (Amaxa). Briefly, RAW264.7 cells were grown to confluence in RPMI 1640 medium supplemented with 10% FBS. Cells were collected and washed once with RPMI 1640 medium and resuspended with the electroporation buffer (Amaxa). After electroporation, the cells were plated on 6-well plates and the transfection efficiency was monitored by fluorescent microscopy. Over 60% cells were GFP-positive. Transient transfection into HEK293 cells was performed using Lipofectamine 2000 according to the manufacturer's instruction.

For promoter analysis, Raw264.7 cells were seeded into 12-well plates and transfected with FuGENE 6 transfection reagent (Roche Applied Science) following the manufacturer's instruction. The total amount of plasmid DNA was kept constant within each experiment. Luciferase activity was measured by the luciferase assay system (Promega) and normalized to β-galactosidase activity by co-transfecting the pCMV-βgal plasmid in all experiments. All transfections were performed in triplicate and repeated at least two times.

RNA Isolation and Northern Blot—Total RNA was isolated from cells using RNA STAT-60 reagent (Tel-Test, Friendswood, TX) following the manufacturer's instruction. 15 μg of total RNA was denatured and electrophoresed on 1% agarose-formaldehyde gels. The uniformity of sample loading was verified by UV visualization of the ethidium bromide-stained gel before transfer to Nylon membrane. The cDNA probes for MCPIP1, 2, 3, and 4 were amplified by PCR using individual cDNA clones from ATCC as templates. ^{32}P-labeled cDNA was prepared using the random priming method (Invitrogen). Hybridization was performed using QuickHyb buffer (Stratagene) at 65 °C for 2 h or overnight. The membranes were then washed once with 2× SSC and once with 0.1× SSC, 1% SDS for 20 min at 65 °C.

Quantitative Real-time PCR—After removing the genomic DNA using DNase I (Ambion), 2.4 μg of total RNA from RAW264.7 or bone marrow-derived macrophage cells was reverse-transcribed to cDNA using a commercially available kit (Applied Biosystems). Quantitative real-time PCR was performed with iCycler Thermal Cycler (Bio-Rad) using 2× SYBR Green master mix (Bio-Rad). Forty cycles were conducted as follows: 95 °C for 30 s, 60 °C for 30 s, preceded by 1 min at 95 °C for polymerase activation. Primer sequences for all genes we measured in this report are available upon request. Quantification was performed by the delta cycle time method, with β-actin used for normalization.

Protein Isolation and Western Blot—After washing twice with PBS, cells were gently scraped with a rubber policeman into 5 ml of ice-cold PBS and centrifuged at 1,000 × *g* for 5 min at 4 °C. Cells from each 10-cm dish were then resuspended and lysed in 0.5 ml of lysis buffer containing 50 mM NaH_2PO_4, pH 7.6, 250 mM NaCl, 50 mM NaF, 10 mM imidazole, 0.5% Nonidet P-40, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. The cell lysate was left on ice for ~20 min and then soni-

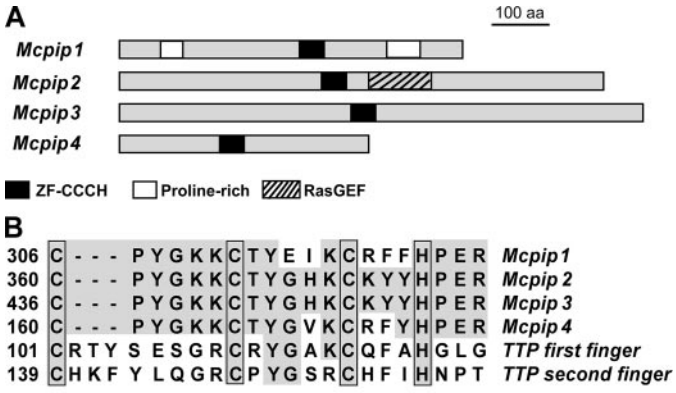
cated and centrifuged at 10,000 × *g* for 10 min at 4 °C. Nuclear and cytoplasmic extracts were prepared using NE-PER Nuclear and Cytoplasmic Extraction kit (Pierce) following the manufacturer's instruction. Protein concentrations were determined by the Bradford method (Bio-Rad) with bovine serum albumin as the standard. For Western blotting, proteins (50 μg) were separated by SDS-PAGE and transferred onto nitrocellulose membranes in transfer buffer containing 0.1% SDS. The membranes were blocked with 5% nonfat dry milk in 0.05% Tween 20 in Tris-buffered saline (TTBS) for 2 h and incubated with the primary antiserum at a 1:1,000 dilution in the blocking buffer for 1 h. After being washed with TTBS three times for 10 min each, the membranes were incubated with a 1:2,000 dilution of secondary antibody in TTBS for 1 h. Following three 10-min washes with TTBS, membranes were incubated with SuperSignal West Pico Chemiluminescent Substrate (Pierce) and exposed to x-ray film.

Immunocytochemistry—THP-1 cells were grown on glass coverslips under the conditions described above. After differentiation into macrophages, the cells were treated with or without LPS (1 μg/ml) for 16 h. The cells were then washed with ice-cold PBS three times before being fixed at room temperature for 10 min in 4% (w/v) paraformaldehyde in PBS. Cells were again washed with PBS twice and permeabilized with 0.2% (v/v) Triton X-100 in TBS at room temperature for 10 min. The cells were incubated at 4 °C overnight in anti-MCPIP1 serum (1:200 dilution) and 1% normal goat serum in 0.1 M sodium phosphate buffer, pH 7.4. Following two washes with 0.2% (w/v) Triton X-100 in TBS, the cells were incubated at room temperature for 1 h in goat anti-rabbit IgG rhodamine-conjugated secondary antibody (1:200 dilution; Chemicon) and 25% normal goat serum in TBS. The cells were rinsed twice with PBS for 10 min each time before being mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA). The slides were examined and imaged with a Nikon fluorescence microscope (Zeiss, Thornwood, NY).

Determination of TNFα, IL-6, and NO Production—NO release was spectrophotometrically determined by measuring the accumulation of nitrite in the medium 8 h after cell activation using Griess Reagent System (Promega). TNFα and IL-6 were measured using enzyme-linked immunosorbent assay kits (eBioscience).

Ac-LDL Uptake—Raw264.7 cells were transfected with pMCPIP1 and pEGFP-N1. 24 h later the cells were treated with Dil-labeled ac-LDL (Biomedical Technologies, Inc.) for the time periods indicated, subsequently washed twice with PBS, and lysed in isopropanol. After sonication followed by 10 min of centrifugation (13,000 × *g*), Dil-labeled ac-LDL content was measured by fluorometry. For microscopy, cells were cultured on glass slides and treated with Dil-labeled ac-LDL.

mRNA Stability Assay—Two approaches were used to measure the mRNA stability. First, Raw264.7 cells were transfected with pMCPIP1 and pEGFP-N1. After 8 h of stimulation with LPS (1 μg/ml), transcription was stopped by adding 5 μg/ml actinomycin D. The cells were harvested and RNA was isolated at different time points as indicated. mRNA level was measured by Northern blot. Second, HeLa Tet-off cells were transfected with βWt or βWt-TNFα reporters and pMCPIP1 or pEGFP-N1 in the presence of 50 ng/ml doxycycline using



C

Name	Other name	Protein (aa)	mRNA (bp)	Chr.	Access number
Human					
MCPIP1	ZC3H12A	599	2697	1	NM_025079
MCPIP2	ZC3H12B	772	7183	X	NM_001010888
MCPIP3	ZC3H12C	852	8807	11	NM_033390
MCPIP4	ZC3H12D	527	2384	6	NM_207360
Mouse					
Mcpip1	Zc3h12a	596	2775	4	NM_153159
Mcpip2	Zc3h12b	835	3365	X	NM_001034907
Mcpip3	Zc3h12c	903	6133	9	XM_993709
Mcpip4	Zc3h12d	430	4095	10	NM_172785

FIGURE 1. **Identification of the MCPIP family.** *A*, schematic representation of the structures of the mouse MCPIP protein family. The conserved domains were indicated. *B*, the amino acid sequences of the CCCH-type zinc finger from the mouse MCPIP family and TTP were aligned by consensus ClustalW sequence alignment using DNASTAR. The amino acids CCCH have been *boxed*, and the other identical amino acids have been *shaded*. *C*, bioinformatic information of both human and mouse MCPIP family was summarized from the NCBI data base. The name for each gene was designated in this study. The other name appeared in the data base. *Chr.*, chromosome.

FuGENE 6 transfection reagent (Roche Applied Science). 24 h later, a transcription pulse from the reporter mRNA expression plasmid was initiated by washing cells with PBS and feeding with 2 ml of Dulbecco's modified Eagle's medium/10% FBS, containing no doxycycline. 6 h later, 3 μg/ml of doxycycline was added to stop transcription. The cells were harvested and RNA was isolated at different time points as indicated. β-Globin mRNA level was measured by Northern blot (12).

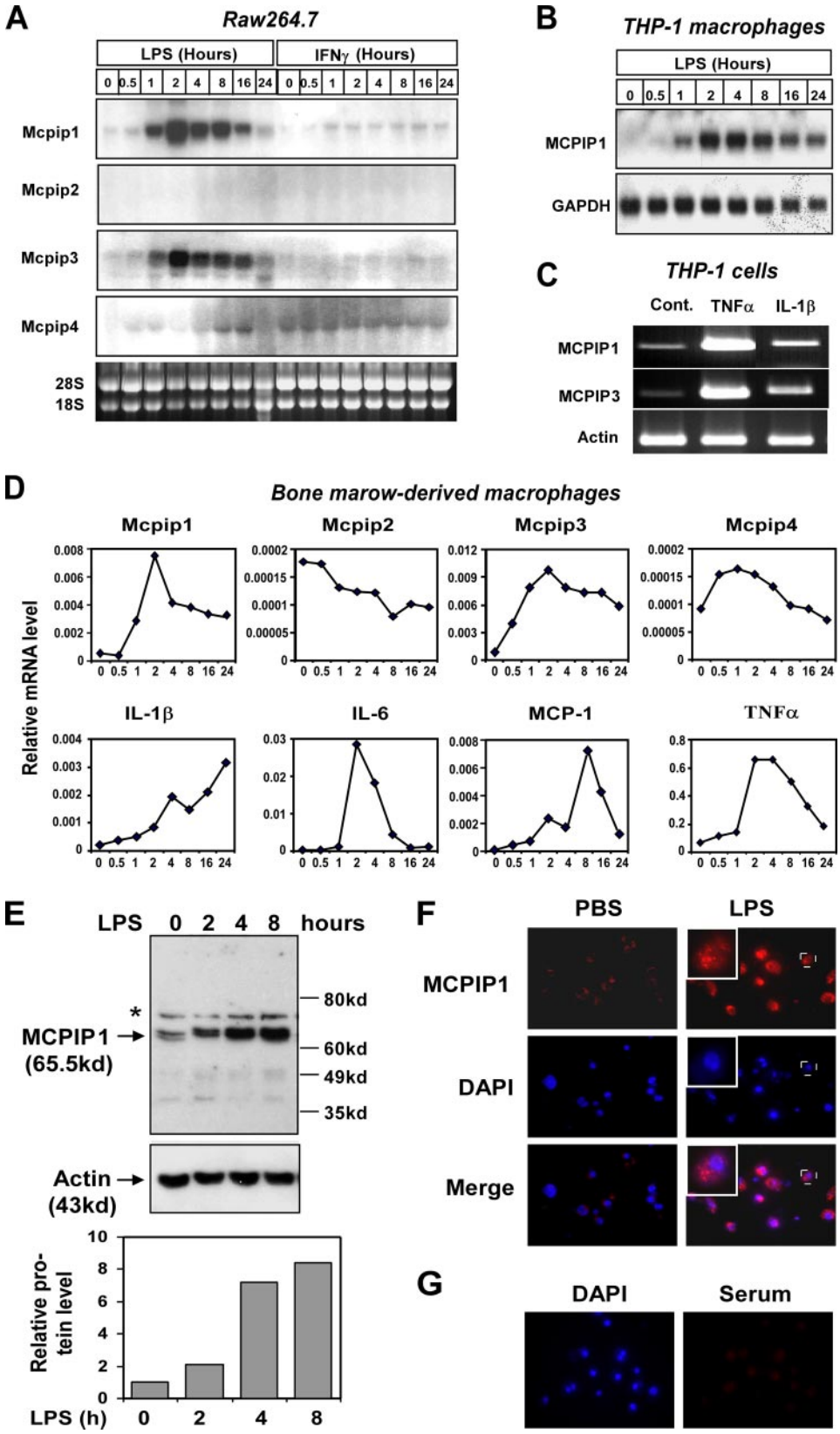
Short Interference RNA—The pre-designed short interference RNA targeting to mouse MCPIP1 (ID number 170484) as well as its negative control were purchased from Ambion (Austin, TX). The short interference RNA was transfected into Raw264.7 cells by electroporation using Amaxa Electroporation Unit following the manufacturer's instruction. 24 h later, the cells were treated with or without LPS (1 μg/ml) for 8 h. The cells were then harvested and RNA was isolated to assess for MCPIP1 knock down and target gene expression.

Statistics—Data were expressed as mean ± S.D. For comparison between two groups, the unpaired Student's test was used. For multiple comparisons, analysis of variance followed by unpaired Student's test was used. A value of $p < 0.05$ was considered significant.

RESULTS

Identification of the MCPIP Protein Family—MCP-1 not only attracts monocytes from the blood stream into the vascular wall but also actively regulates gene expression and contributes to

the pathogenesis of inflammatory diseases. In an effort to search MCP-1-induced genes in human monocytes using gene microarray analysis, we previously identified a highly MCP-1-induced gene, which was designated MCPIP (as MCP-1 Induced Protein) (4). This gene encodes a novel CCCH-zinc finger-containing protein. By searching similar sequences in GenBank™, we have found that four members belong to this protein family that are designated as MCPIP1, 2, 3, and 4 and encoded by four genes, *Zc3h12a*, *Zc3h12b*, *Zc3h12c*, and *Zc3h12d*, respectively. The obvious feature of this protein family is that they all contain a single CCCH-type zinc finger (Fig. 1*A*). In addition to the central CCCH-zinc finger, MCPIP1 contains two proline-rich domains at the N- and C-terminals, which may mediate interaction with Src homology 3 domain-containing proteins. MCPIP2 has a RasGEF domain at the C-terminal of the zinc finger motif, suggesting its potential involvement in signal transduction. No other specific motifs are observed in MCPIP3 and 4. It is worth noting that the zinc finger in MCPIP family is $C(X)_5C(X)_5C(X)_3H$ type and very conserved among this family, which is somewhat different from the two fingers in TTP that were $C(X)_8C(X)_5C(X)_3H$ type (Fig. 1*B*). The bioinformatics of human and mouse MCPIP family was summarized in Fig. 1*C*. Their mRNA length represents the longest mRNA in GenBank™, which may not be the actual full length. The protein weight is predicted and not yet confirmed experimentally. Proteins containing CCCH-zinc finger have been identified from metazoan organisms, yeast, and plant. It seems that the MCPIP family is much conserved during evolution as we identified cDNA sequences that encode proteins highly similar to MCPIPs from *Drosophila melanogaster*, *Caenorhabditis elegans* to *Mus musculus* and *Rattus norvegicus*. The homology between human and mouse MCPIPs varies from 81 to 92%. The amino acid identity between different mouse MCPIP family members is ~40%, but the homology of their zinc finger motif is >80%. Phylogenetic analysis indicates that MCPIP2 and MCPIP3 are more closely related to each other than to other MCPIP family members (data not shown).



Induction of the MCPIP Family during Macrophage Activation—Macrophage activation is an essential cellular process underlying innate immunity, enabling the body to combat bacteria and other pathogens. In addition to host defense, activated macrophages play a central role in atherogenesis, autoimmunity, and a variety of inflammatory diseases. To explore whether the MCPIP family is involved in the regulation of macrophage activation, we examined the expression of MCPIPs during LPS- or IFNγ-induced macrophage activation, which are the most studied two inducers for macrophage activation. As shown in Fig. 2*A*, Northern blot revealed that both MCPIP1 and MCPIP3 were highly induced in LPS-, but not IFNγ-, stimulated murine macrophage cell line Raw264.7. Their expression was raised after 1 h of LPS treatment, peaked at 2 h by >20-fold above basal line, and was sustained at a high level for at least 16 h. MCPIP4 was also induced to some extent by LPS over a 16-h time course, whereas MCPIP2 was not induced at all during the time periods of both LPS and IFNγ stimulation. LPS also stimulated expression of MCPIP1 in THP-1-derived macrophages over the time course of 1 to 24 h with a pattern similar to Raw264.7 (Fig. 2*B*). Both MCPIP1 and MCPIP3 were also induced by endogenous cytokines TNFα and IL-1β in THP-1 cells (Fig. 2*C*). We further explored the effect of LPS on the expression of MCPIPs in mouse primary bone marrow-derived macrophages. Consistent with the results from Raw264.7, both MCPIP1 and MCPIP3 transcripts increased a remarkable 25-fold over basal levels at 2 h after LPS stimulation and then declined a little bit but sustained a substantial level for 24 h. The basal levels of both MCPIP2 and MCPIP4 seemed very low and did not change after exposure to LPS in the 24-h time period (Fig. 2*D*). To validate our LPS stimulations, we also assessed the temporal expression of macrophage inflammatory mediators IL-1β, IL-6, MCP-1, and TNFα. Consistent with the previous report, they were all potently induced by ten- to thousands-fold after LPS treatment (Fig. 2*D*). It is interesting to note that with the exception of IL-1β, which constantly increased during the 24 h of stimulation, the mRNA of IL-6, MCP-1, and TNFα reached a peak at 2 or 8 h of stimulation and then declined to basal level at 24 h, suggesting that a negative regulatory mechanism may terminate the proinflammatory signals.

Next we examined macrophage MCPIP1 protein expression in response to LPS. Western blot analysis detected a band around 65.5 kDa, which is the expected protein size of MCPIP1. MCPIP1 protein was increased by 8-fold within 8 h of LPS stimulation in THP-1-derived macrophages (Fig. 2*E*). MCPIP1 protein expression was further studied using immunofluorescence microscopy. Treatment of THP-1 macrophages with LPS led to a marked increase in MCPIP1 protein expression. MCPIP1 protein seems predominantly localized in the cytoplasm (Fig. 2*F*). No specific staining was seen by control serum (Fig. 2*G*).

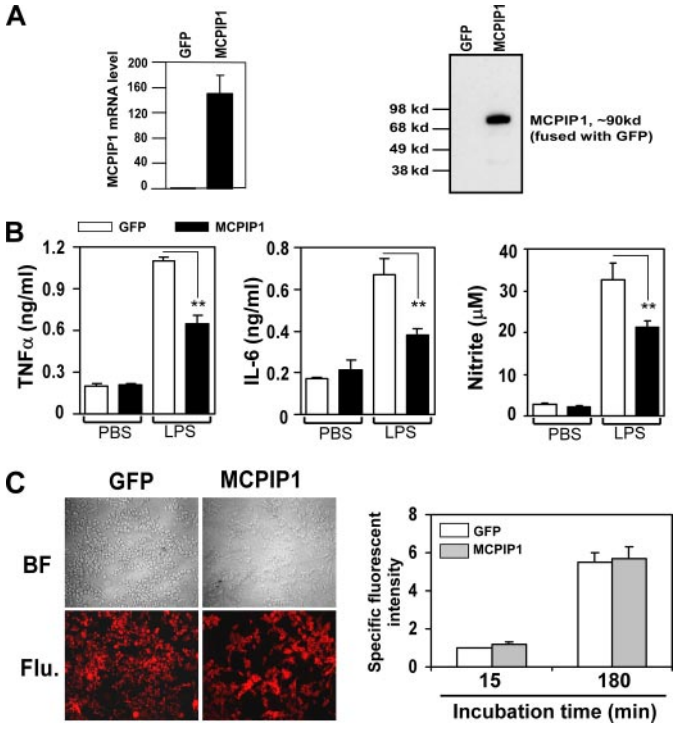
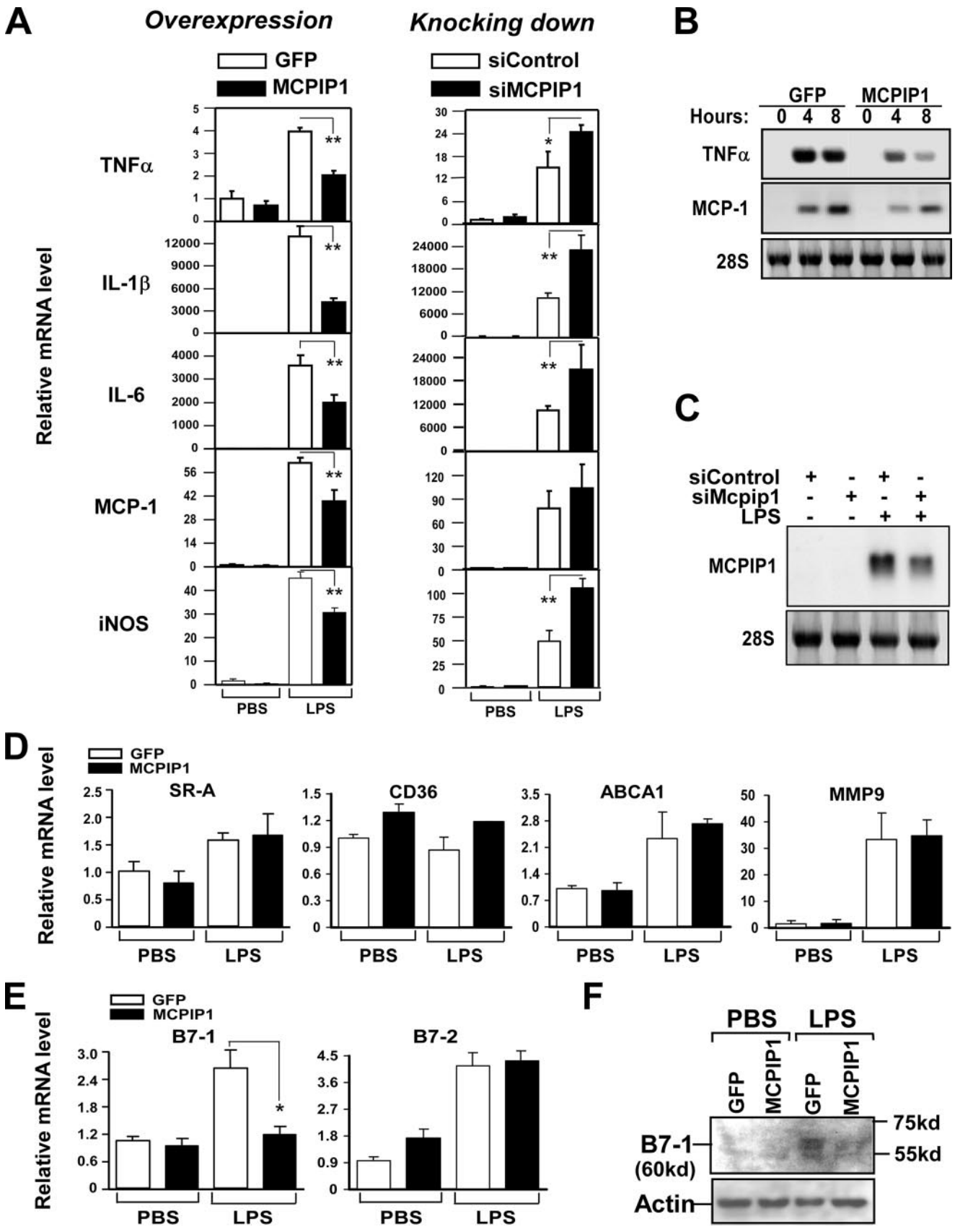


FIGURE 3. **Overexpression of MCPIP1 inhibits LPS-induced TNFα, IL-6, and NO_2^- production but does not affect lipid uptake.** *A*, Raw264.7 cells were transiently transfected with pEGFP-N1 or pMCPIP1 by electroporation (Amaxa). The overexpression of MCPIP1 in the transfected cells was monitored by quantitative PCR (*left*) and Western blotting (*right*). *B*, the transfected cells were quiescent for 24 h and then treated with PBS or 1 μg/ml LPS for 6 h. The cultured medium was harvested for measurement of TNFα, IL-6, and NO production. Data are represented as mean ± S.D., $n = 3$. **, $p < 0.001$. *C*, the transfected Raw264.7 cells were treated with Dil-labeled ac-LDL for 6 h and visualized by fluorescent microscopy (*left*) or incubated with Dil-ac-LDL for 15 and 180 min and determined by fluorometry (*right*). Data are represented as mean ± S.D., $n = 3$.

MCPIP1 Negatively Regulates LPS-induced Macrophage Activation but Does Not Affect Lipid Uptake—MCPIP1 was significantly induced during LPS-induced macrophage activation, suggesting that it may be involved in the regulation of this important process. To test this idea, the murine macrophage cell line Raw263.7 cells were transiently transfected with pMCPIP1 or pEGFP-N1 by electroporation. Overexpression of MCPIP1 was confirmed by microscopy as well as quantitative

FIGURE 2. **Expression of MCPIP family members is induced by LPS in macrophages.** *A*, Raw264.7 cells were treated with LPS (1 μg/ml) or IFNγ (2 ng/ml) for different times as indicated. Total RNA (15 μg/lane) was analyzed by Northern blotting using ^{32}P-labeled cDNA probes. Ethidium bromide-stained RNA image served as loading control. *B*, THP-1 cells were differentiated into macrophages by treatment with phorbol 12-myristate 13-acetate (100 ng/ml) for 5 days. After 24 h of quiescence, the cells were treated with LPS (1 μg/ml) for the indicated times. Total RNA was isolated, and Northern blotting was performed to detect MCPIP1 expression. The same membrane was probed with glyceraldehyde-3-phosphate dehydrogenase and served as loading control. *C*, THP-1 cells were quiescent for 16 h and then treated with TNFα (10 ng/ml) or IL-1β (10 ng/ml) for 8 h. mRNA levels of MCPIP1 and MCPIP3 were measured by reverse transcription PCR, and β-actin served as loading control. *D*, monocyte progenitors from C57BL6/J mice were obtained and differentiated into primary macrophages. Primary macrophages were exposed to LPS (1 μg/ml) and harvested at the indicated time points. Samples were processed and subjected to quantitative PCR analysis. Data are from three independent experiments and normalized to β-actin expression. *E*, THP-1 cells were differentiated into macrophages as described above. After 24 h of quiescence, the cells were treated with LPS (1 μg/ml) for the indicated times. Cell lysates were prepared and MCPIP1 protein level was evaluated by Western blotting (40 μg protein/lane) as described under "Materials and Methods." The intensity of bands was quantified by AlphaImage 2200 (AlphaInnotech), and the normalized protein levels are shown (*bottom*). *, nonspecific bands. *F* and *G*, THP-1-derived macrophages were treated with PBS or 1 μg/ml LPS for 16 h. The cells were then permeated and stained with antibody to MCPIP1 (*B*) or control serum (*C*) and counterstained with 4′,6′-diamidino-phenylindole (*DAPI*).



PCR and Western blot (Fig. 3*A*). In cells overexpressing MCPIP1, we observed a marked reduction in the LPS-induced production of TNFα, IL-6, and NO_2^- (Fig. 3*B*). However, it was noted that overexpression of MCPIP1 did not affect Dil-ac-LDL uptake (Fig. 3*C*).

MCPIP1 Negatively Regulates LPS-induced Inflammatory Gene Expression but Does Not Affect the Cell Surface Marker Expression in Macrophages—Next, we further investigated the effect of MCPIP1 on LPS-stimulated gene expression. As shown in Fig. 4*A*, LPS dramatically induced inflammatory cytokine (TNFα, IL-1β, IL-6, and MCP-1) expression by several- to thousands-fold. Interestingly, MCPIP1 overexpression significantly blunted the induction of TNFα, IL-1β, IL-6, and MCP-1 by LPS. Conversely, knocking down of MCPIP1 using specific short interference RNA strongly augmented the induction of TNFα, IL-1β, IL-6, and MCP-1 by LPS (Fig. 4*A*). The efficient knocking down of MCPIP1 by siRNA was confirmed by Northern blotting (Fig. 4*C*). We further observed the expression changes of TNFα and MCP-1 during a time period of LPS stimulation in green fluorescent protein- or MCPIP1-transfected cells by Northern blotting. As shown in Fig. 4*B*, significant decrease in TNFα and MCP-1 mRNA was observed in MCPIP1 overexpressed cells after 4 h of stimulation. Consistent with the functional examination above, MCPIP1 negatively regulated iNOS expression but did not affect the expression of scavenger receptor A, CD36, ABCA1, and matrix metalloproteinase-9 in LPS-treated macrophages (Fig. 4*D*). Interestingly, although two members of costimulatory molecule B7 family, B7-1 and B7-2, were also substantially induced by LPS in macrophages, overexpression of MCPIP1 inhibited B7-1, but not B7-2, induction (Fig. 4, *E* and *F*).

MCPIP1 Does Not Affect mRNA Degradation of Inflammatory Cytokines—The expression of inflammatory cytokines, including TNFα, IL-1β, IL-6, and MCP-1, is tightly regulated at both transcriptional and posttranscriptional levels. It is noted that the TTP family, a well studied CCCH-zinc finger protein family, can bind to the ARE at the 3′-untranslated region of TNFα mRNA and promote its decay (7–9). To explore the mechanisms of the MCPIP1 effect on inflammatory cytokines, we first evaluated the potential influence of MCPIP1 on the mRNA stability of TNFα and MCP-1 in LPS-stimulated Raw264.7 cells. In the experiment shown in Fig. 5*A*, the cells were stimulated with LPS (1 μg/ml; Sigma) for 8 h and then treated with actinomycin D (5 μg/ml; Sigma). The RNA was isolated at different time points as indicated. Surprisingly, although both TNFα and MCP-1 mRNA levels were significantly decreased in MCPIP1-overexpressing cells, the half-lives of both TNFα and MCP-1 mRNA were barely affected by overexpression of MCPIP1 (48 and 110 min *versus* 44 and 100 min, respectively). These results cannot explain why MCPIP1 downregulate the inflammatory cytokine expression. However, there is a possibility that MCPIP1 may work with one or more other inducible proteins to promote mRNA decay and that actinomycin D may block the expression of those proteins to diminish their effect. To exclude this possibility, we performed an ARE-mediated mRNA decay assay in human HeLa Tet-off cells as described previously (12). Basically, two β-globin reporters, βWt (containing human β-globin gene) or βWt-TNFα (containing β-globin gene and the potent ARE from TNFα mRNA in the 3′-untranslated region), were co-transfected with pMCPIP1 or pEGFP-N1 into the HeLa Tet-off cell line (both the reporters and the HeLa Tet-off cell line were kindly provided by Dr. Jens Lykke-Andersen). Transcription of the β-globin mRNA is controlled by a tetracycline regulatory promoter that allows for measurement of the mRNA decay rate in pulse-chase experiments in human HeLa Tet-off cells. The results in Fig. 5*B* showed that MCPIP1 did not affect the ARE-containing β-globin mRNA decay. These results indicate that MCPIP1 does not directly regulate TNFα mRNA stability.

MCPIP1 Negatively Regulates the TNFα and iNOS Promoter and Blocks LPS-induced NF-κB Activation—We then asked whether MCPIP1 regulate the promoter activity of the inflammatory genes. For these studies, we used a −1.2-kb fragment of the mouse TNFα promoter and −1.5-kb fragment of the mouse iNOS promoter. These promoters contain all of the regulatory elements necessary for LPS responsiveness *in vivo* (13, 14). As shown in Fig. 6*A*, forced expression of MCPIP1 dose dependently inhibited LPS-induced promoter activity of TNFα and iNOS. As NF-κB is a key transcription factor to regulate both TNFα and iNOS promoters, we tested whether MCPIP1 would also inhibit p65-induced TNFα and iNOS promoter activation. As shown in Fig. 6*B*, MCPIP1 also significantly decreased both p65-induced TNFα and iNOS promoter activity in a dose-dependent manner. Finally, we asked whether MCPIP1 can block LPS-induced NF-κB activation. As expected, LPS or p65 markedly induced NF-κB mini promoter activation (Fig. 6*C*), whereas MCPIP1 strongly blocked LPS- or p65-induced NF-κB activation. This effect was specific, because MCPIP1 did not block peroxisome proliferator-activator receptor γ-induced PPRE promoter activation (Fig. 6*C*). These results strongly suggest that MCPIP1 may function as a feedback inhibitor for LPS-induced NF-κB signaling, by which it negatively regulates inflammatory gene expression as well as macrophage activation.

DISCUSSION

In an effort to search MCP-1-induced genes in human monocytes, we previously identified a highly MCP-1-induced pro-

FIGURE 4. **MCPIP1 regulates a distinct group of inflammatory gene expression.** *A*, in the overexpression experiments, Raw264.7 cells were transiently transfected with pEGFP-N1 or pMCPIP1 by electroporation (Amaxa). In the knocking down experiments, Raw264.7 cells were transiently transfected with short interfering RNA targeting MCPIP1 (*siMCPIP1*) or nonspecific short interfering RNA (*siControl*) by electroporation (Amaxa). The transfected cells were quiescent for 24 h and then treated with PBS or 1 μg/ml LPS for 8 h. RNA was isolated for quantitative PCR analysis. *, $p < 0.05$; **, $p < 0.001$. *B*, RNA samples from the overexpression experiments were further subjected to Northern blot analysis. The image for 28 S RNA served as loading control. *C*, RNA samples from the knocking down experiments were further subjected to Northern blot analysis. The image for 28 S RNA served as loading control. *D* and *E*, the RNA samples from overexpression experiments were analyzed by quantitative PCR for some other gene expression as indicated. Data were normalized by β-actin and are represented as mean ± S.D., $n = 3$. *F*, Raw264.7 cells were transiently transfected with pEGFP-N1 or pMCPIP1 by electroporation (Amaxa). The transfected cells were quiescent for 24 h and then stimulated with LPS (1 μg/ml) for 26 h. Protein was isolated and analyzed by Western blot using goat anti-B7-1 polyclonal antibody.

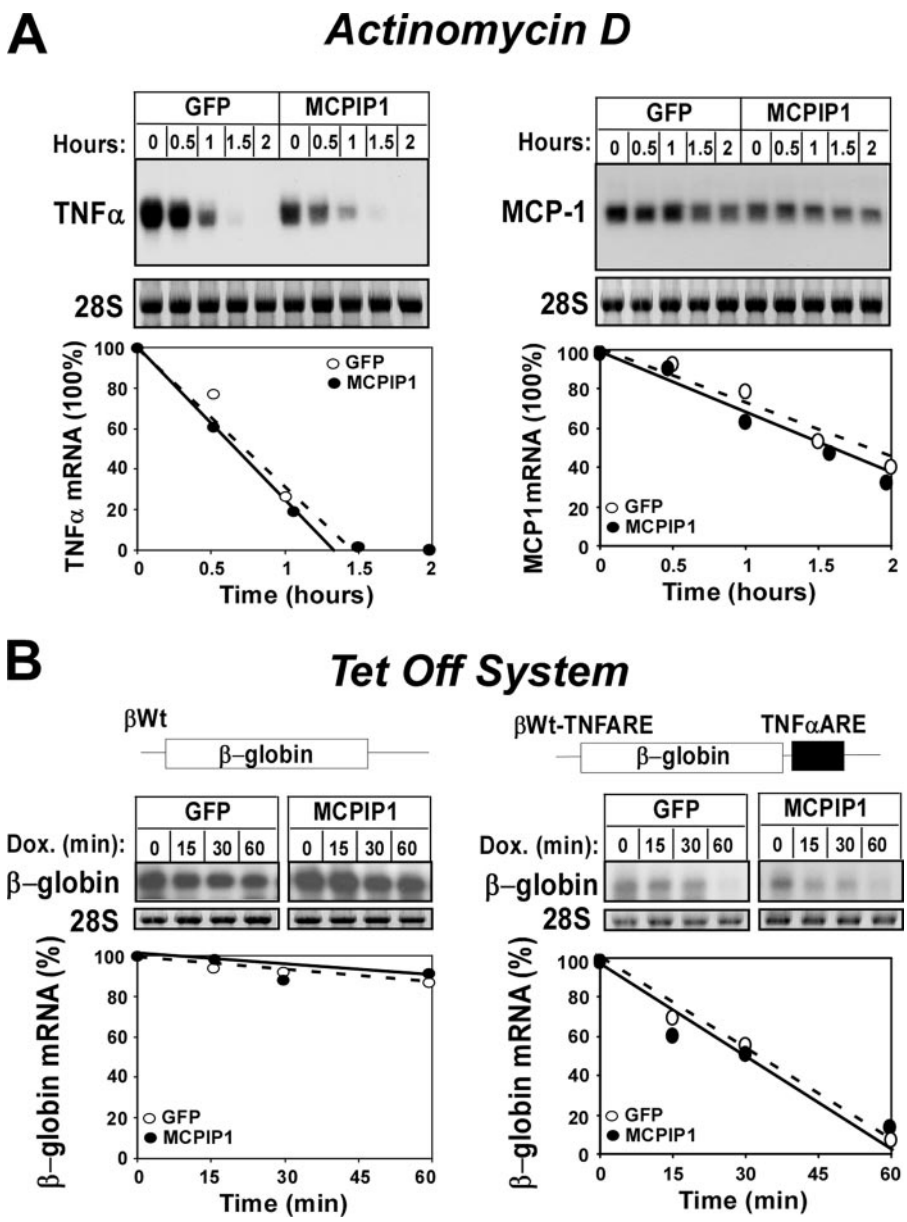


FIGURE 5. **Effect of MCPIP1 on TNFα and MCP-1 mRNA stability in macrophages.** *A*, Raw264.7 cells were transiently transfected with pEGFP-N1 or pMCPIP1 by electroporation. After 24 h, the cells were quiescent for 18 h and then treated with LPS (1 μg/ml) for 8 h, and then actinomycin D (5 μg/ml) was added to stop transcription. RNA was harvested after different time points as indicated. TNFα and MCP-1 mRNA levels were examined by Northern blot. The intensity of bands was quantified by AlphaImage 2200 (AlphaInnotech). The value of 0 time point was set as 100%, and the values of the other points were transformed according to 0 point. The data from three independent experiments were averaged and plotted in the figures at the bottom of each image. *B*, HeLa Tet-off cells were transfected with βWt or βWt-TNFARE reporter with or without pEGFP-N1 or pMCPIP1 as indicated by FuGENE 6, in the presence of doxycycline. After 24 h, the cells were washed with PBS and incubated with medium without doxycycline for 6 h, and then doxycycline (3 μg/ml) was added to stop transcription of the reporter. RNA was harvested after different time points as indicated. β-Globin mRNA level was examined and analyzed by the same procedure as above.

tein, MCPIP. In this study, we have found that there are four members, MCPIP1, 2, 3, and 4, belonging to this subfamily. The obvious feature of this protein family is that they all contain a single CCCH-type zinc finger motif and their transcripts were enriched in immune organ as well as other organs (data not shown). As a start, here we characterized MCPIP1 as a negative regulator of LPS-dependent macrophage activation.

CCCH-type zinc finger is unusual in mammalian genomes. Searching the protein data base, we found 58 CCCH-type zinc finger proteins in human and murine genomes.[3] Most of them were not characterized yet. Among the characterized CCCH-type zinc finger proteins, most are capable of binding to RNA and regulate mRNA processing, including mRNA maturation, export, modification, and turnover (15, 16). TTP is the prototype of a family of CCCH-zinc finger proteins that contain two $(CX)_8(CX)_5(CX)_3$H-type zinc finger motifs. Although initially discovered as a gene that could be induced rapidly and transiently by the stimulation of fibroblasts with growth factors and mitogens (17), it is now known that TTP can bind to AU-rich elements in mRNA, leading to the removal of the poly(A) tail from that mRNA and increased rates of mRNA turnover. TTP-deficient mice developed a systemic inflammatory syndrome with severe polyarticular arthritis and autoimmunity, as well as medullary and extramedullary myeloid hyperplasia due to excess circulating TNFα, resulting from the increased stability of the TNF mRNA and subsequent higher rates of secretion of the cytokine (18). We originally hypothesized that MCPIP1 may also down-regulate the expression of proinflammatory genes through promoting their mRNA decay. Two approaches were used to examine the effect of MCPIP1 on TNFα mRNA decay and show that MCPIP1 does not affect TNFα mRNA stability at all. Therefore, the MCPIP family may function in a different way than other CCCH-zinc finger families.

We then looked at the impact of MCPIP1 on LPS-induced TNFα promoter activation. Interestingly, forced expression of MCPIP1 significantly inhibited LPS-stimulated TNFα promoter activation. Similar results were observed in the iNOS promoter. These results may explain why overexpression of MCPIP1 dramatically attenuated LPS-induced expression of TNFα and iNOS as well as LPS-induced NO_2^- synthesis and TNFα production in macrophages. The major direct signal pathway for macrophage activation by LPS is the NF-κB signal-

[3] J. Liang and M. Fu, unpublished data.

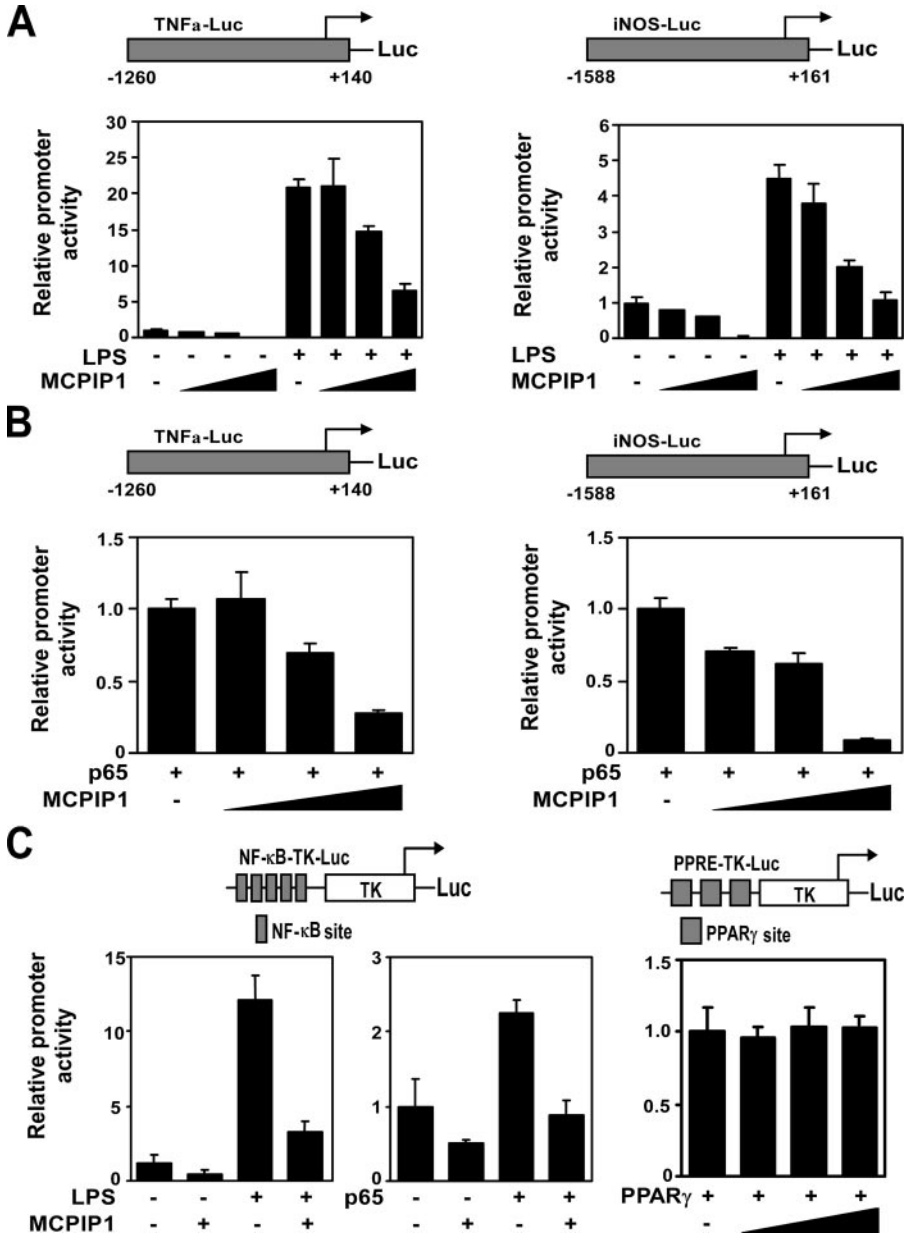


FIGURE 6. **MCPIP1 negatively regulates the TNFα and iNOS promoters and blocks LPS-induced NF-κB activation.** *A*, Raw264.7 cells were co-transfected with a luciferase reporter plasmid under transcriptional control of the TNFα or iNOS promoters and increasing amounts of pMCPIP1 (0, 5, 50, and 500 ng/well). After being quiescent for 24 h, cells were treated with or without LPS (100 ng/ml) as shown and collected for analysis of reporter gene activity 24 h later. *B*, Raw264.7 cells were co-transfected with the TNFα or iNOS reporter, p65, and increasing amounts of pMCPIP1 (0, 5, 50, and 500 ng/well). *C*, Raw264.7 cells were co-transfected with an NF-κB or PPRE mini reporter and increasing amounts of pMCPIP1 (0, 5, 50, and 500 μg/well). NF-κB reporter was stimulated by LPS (100 ng/ml) or induced by co-transfection with p65, whereas PPRE reporter was stimulated by proliferator-activator receptor γ co-transfection and treatment with rosiglitazone (1 μM). Cells were collected for analysis of reporter gene activity 24 h later.

ing pathway (19). To further understand the molecular mechanisms by which MCPIP1 regulates the proinflammatory response of macrophages to LPS, we observed the effect of MCPIP1 on LPS-dependent NF-κB activation. Overexpression of MCPIP1 inhibited LPS-triggered NF-κB activation, but not rosiglitazone-induced proliferator-activator receptor γ activation, suggesting that MCPIP1 may be a novel inhibitor of the NF-κB activation pathways (Fig. 6). In addition, overexpression of MCPIP1 inhibited p65-induced activation of the TNFα promoter, iNOS promoter, and NF-κB mini promoter, suggesting that MCPIP1 may directly target p65. Interestingly, although LPS also significantly induced expression of the macrophage surface protein scavenger receptor A and secreted enzyme matrix metalloproteinase 9, overexpression of MCPIP1 did not affect their induction at all. Because the major signal pathway contributing to scavenger receptor A and matrix metalloproteinase 9 induction is p38 kinase-activator protein-1 (AP-1) pathway (14, 20), we reason that MCPIP1 may selectively target LPS-induced NF-κB signal pathway but not AP-1 signaling. This notion is further supported by the observation of two costimulatory molecules, B7-1 and B7-2. B7-1 and B7-2 are related immunoglobulin supergene family members that are expressed by multiple cell types, including monocytes, macrophages, B cells, and T cells, following activation (21). Both B7-1 and B7-2 were induced by LPS in macrophages. B7-1 induction is dependent on NF-κB signaling, whereas B7-2 induction is independent of NF-κB signaling (22, 23). We observed that MCPIP1 blocked B7-1, but not B7-2, induction by LPS, suggesting MCPIP1 may function as a specific inhibitor for NF-κB signaling.

Considering that MCPIP1 is induced by a range of inflammatory stimuli such as LPS, TNFα, IL-1β, and MCP-1, we conclude that MCPIP1 may function as a feedback inhibitor of macrophage activation by targeting to NF-κB signaling. Currently, the detailed mechanisms whereby MCPIP1 inhibits NF-κB activation are unknown. Several NF-κB inhibitors recently identified, such as suppressor of cytokine signaling 1 (SOCS1), A-20, Bcl-3, FLN29, and PDLIM2, down-regulate NF-κB signaling by targeting to NF-κB ubiquitination pathway (24–28). At least two CCCH-zinc finger-containing proteins, Roquin and markrin-1, function as ubiquitin ligase (11, 29). A study to determine whether MCPIP1 also target the NF-κB ubiquitination system is under way.

Currently, the function of the other three members in the MCPIP family remains to be determined. MCPIP2 was highly expressed in brain, thymus, and testis under normal conditions (data not shown) but not expressed in LPS- or IFNγ-activated

macrophages. Besides a central CCCH-zinc finger motif, it has a RasGEF domain, suggesting that it may be involved in Ras-mediated signal transduction. MCPIP3 has a different tissue expression pattern than MCPIP1, but they have a similar induction in LPS-induced macrophage activation. MCPIP4 is specifically abundant in spleen, intestine, and colon and also induced to some extent by LPS in Raw264.7 cells. Further studies are needed to characterize their function.

It is well established that uncontrolled inflammation does not benefit organisms but instead causes tissue impairment. Many mechanisms have been evolved to control the intensity of pathogen-initiated inflammatory response through negative feedback. In this sense, the MCPIP protein family may be critically involved in the regulation of macrophage activation and implicated in pathogenesis of inflammatory diseases. Our previous works have demonstrated that MCPIP1 transcript level was much higher in explanted human hearts with ischemic heart disease (4) and adipose tissues from diet-induced obese mice,[4] both of the conditions having inflammatory status. Future studies will help to elucidate the importance and mechanisms of the MCPIP family in human inflammatory diseases.

Acknowledgments—We thank Dr. Jifeng Zhang (University of Michigan) and Dr. Jianguo Liu (Cornell University) for helpful discussion. We are grateful to Dr. Jens Lykke-Andersen (University of Colorado) for providing βWt and βWt-TNFα plasmids and the HeLa Tet-off cell line. We also thank Dr. Dmitry Kuprash (Russian Academy of Science) for the gift of TNFα-Luc reporter. Finally, we thank Yue Liang for proofreading the manuscript and Dr. Zhelyabouska for technical help.

REFERENCES

1. Burke, B., and Lewis, C. E. (eds) (2002) *The Macrophage* 2nd Ed., pp. 1–3, Oxford University Press, Oxford
2. Ma, J., Chen, T., Mandelin, J., Ceponis, A., Miller, N. E., Hukkanen, M., Ma, G. F., and Konttinen, Y. T. (2003) *Cell. Mol. Life Sci.* **60,** 2334–2346
3. Beutler, B., and Rietschel, E. T. (2003) *Nat. Rev. Immunol.* **3,** 169–176
4. Zhou, L., Azfer, A., Niu, J., Graham, S., Choudhury, M., Adamski, F. M., Younce, C., Binkley, P. F., and Kolattukudy, P. E. (2006) *Circ. Res.* **98,** 1–10
5. Mackay, J. P., and Crossley, M. (1998) *Trends Biochem. Sci.* **23,** 1–4
6. Matthews, J. M., and Sunde, M. (2002) *IUBMB Life* **54,** 351–355
7. Carrick, D. M., Lai, W. S., and Blackshear, P. J. (2004) *Arthritis. Res. Ther.* **6,** 248–264
8. Lai, W. S., Carballo, E., Strum, J. R., Kennington, E. A., Phillips, R. S., and Blackshear, P. J. (1999) *Mol. Cell. Biol.* **19,** 4311–4323
9. Carballo, E., Lai, W. S., and Blackshear, P. J. (1998) *Science* **281,** 1001–1005
10. Gao, G., Guo, X., and Goff, S. P. (2002) *Science* **297,** 1703–1706
11. Vinuesa, C. G., Cook, M. C., Angelucci, C., Athanasopoulos, V., Rui, L., Hill, K. M., Yu, D., Domaschenz, H., Whittle, B., Lambe, T., Roberts, I. S., Copley, R. R., Bell, J. I., Cornall, R. J., and Goodnow, C. C. (2005) *Nature* **435,** 452–458
12. Lykke-Andersen, J., and Wagner, E. (2005) *Genes Dev.* **19,** 351–361
13. Kuprash, D. V., Udalova, I. A., Turetskaya, R. L., Kwiatkowski, D., Rice, N. R., and Nedospasov, S. A. (1999) *J. Immunol.* **162,** 4045–4052
14. Ricote, M., Li, A. C., Willsons, T. M., Kelly, C. J., and Glass, C. K. (1998) *Nature* **391,** 79–82
15. Brown, R. S. (2005) *Curr. Opin. Struct. Biol.* **15,** 94–98
16. Hall, T. M. (2005) *Curr. Opin. Struct. Biol.* **15,** 367–373
17. DuBois, R. N., McLane, M. W., Ryder, K., Lau, L. F., and Nathans, D. J. (1990) *J. Biol. Chem.* **265,** 19185–19191
18. Taylor, G. A., Carballo, E., Lee, D. M., Lai, W. S., Thompson, M. J., Patel, D. D., Schenkman, D. I., Gilkeson, G. S., Broxmeyer, H. E., Haynes, B. F., and Blackshear, P. J. (1996) *Immunity* **4,** 445–454
19. Viatour, P., Merville, M. P., Bours, V., and Chariot, A. (2005) *Trends Biochem. Sci.* **30,** 43–52
20. Woo, C. H., Lim, J. H., and Kim, J. H. (2004) *J. Immunol.* **173,** 6973–6980
21. McAdam, A. J., Schweitzer, A. N., and Sharpe, A. H. (1998) *Immunol. Rev.* **165,** 231–247
22. Zhao, J., Freeman, G. J., Gray, G. S., Nadler, L. M., and Glimcher, L. H. (1996) *J. Exp. Med.* **183,** 777–789
23. Lim, W., Ma, W., Gee, K., Aucoin, S., Nandan, D., Diaz-Mitoma, F., Kozlowski, M., and Kumar, A. (2002) *J. Immunol.* **168,** 1759–1769
24. Yoshimura, A., Naka, T., and Kubo, M. (2007) *Nat. Rev. Immunol.* **7,** 454–465
25. Wertz, I. E., O'Rourke, K. M., Zhou, H., Eby, M., Aravind, L., Seshagirl, S., Wu, P., Wiesmann, C., Baker, R., Boone, D. L., Ma, A., Koonin, E. V., and Dixit, V. M. (2004) *Nature* **430,** 694–699
26. Carmody, R. J., Ruan, Q., Palmer, S., Hilliard, B., and Chen, Y. H. (2007) *Science* **317,** 675–678
27. Mashima, R., Saeki, K., Aki, D., Minoda, Y., Takaki, H., Sanada, T., Kobayashi, T., Aburatani, H., Yamanashi, Y., and Yoshimura, A. (2005) *J. Biol. Chem.* **280,** 41289–41297
28. Tanaka, T., Grusby, M. J., and Kaisho, T. (2007) *Nat. Immunol.* **8,** 584–591
29. Kim, J. H., Park, S.-M., Kang, M. R., Oh, S.-Y., Lee, T. H., Muller, M. T., and Chung, I. K. (2005) *Genes Dev.* **19,** 776–781

[4] A. Azfer, M. Fu, and P. E. Kolattukudy, unpublished data.

A Novel CCCH-Zinc Finger Protein Family Regulates Proinflammatory Activation of Macrophages

Jian Liang, Jing Wang, Asim Azfer, Wenjun Song, Gail Tromp, Pappachan E. Kolattukudy and Mingui Fu

J. Biol. Chem. 2008, 283:6337-6346.
doi: 10.1074/jbc.M707861200 originally published online January 4, 2008
