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Transcription Factors STAT6 and KLF4 Implement Macrophage Polarization via the Dual Catalytic Powers of MCPIP

Nidhi Kapoor,1 Jianli Niu,1 Yasser Saad, Sanjay Kumar, Tatiana Sirakova, Edilu Becerra, Xiaoman Li, and Pappachan E. Kolattukudy

Macrophage polarization plays a critical role in tissue homeostasis, disease pathogenesis, and inflammation and its resolution. IL-4–induced macrophage polarization involves induction of STAT6 and Kruppel-like factor 4 (KLF4), which induce each other and promote M2 polarization. However, how these transcription factors implement M2 polarization is not understood. We report that in murine macrophages MCP-1–induced protein (MCPIP), induced by KLF4, inhibits M1 polarization by inhibiting NF-κB activation and implements M2 polarization using both its deubiquitase and RNase activities that cause sequential induction of reactive oxygen species (ROS), endoplasmic reticulum (ER) stress, and autophagy required for M2 polarization. MCPIP also induces C/EBPβ and PPARγ, which promote M2 polarization. Macrophages from mice with myeloid-targeted overexpression of MCPIP show elevated expression of M2 markers and reduced response to LPS, whereas macrophages from mice with myeloid-specific deletion of MCPIP manifest elevated M1 polarization with enhanced phagoctytic activity. Thus, both in vivo and in vitro experiments demonstrate that the transcription factors STAT6 and KLF4 implement IL-4–induced M2 polarization via the dual catalytic activities of MCPIP. The Journal of Immunology, 2015, 194: 6011–6023.

Abbreviations used in this article: Arg1, arginase 1; ER, endoplasmic reticulum; ES, endogenous species; siRNA, small interfering RNA; TUDC, tauroursodeoxycholate; WT, wild-type.

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and autophagy required for M2 polarization. In this study, we present experimental evidence to support this hypothesis.

We demonstrate that treatment of murine peritoneal macrophages with IL-4 induces MCPiP via induction of KLF4, and that the M2 polarization induced by the IL-4 is mediated via MCPiP. We also demonstrate that macrophages from mice with specific deletion of MCPiP are incapable of IL-4-induced M2 polarization. We show that macrophages from transgenic animals with macrophage-specific overexpression of MCPiP inhibited expression of M1 markers and stimulated expression of M2 markers. Thus, both in vitro and in vivo experiments show that MCPiP plays a critical role in M2 polarization. MCPiP is known to have deubiquitinase and RNAse activities, including anti-Dicer activity. With MCPiP mutants that have only one of the two catalytic activities, we demonstrate that both of these catalytic powers of MCPiP implement the IL-4 induction of differentiation mediated via transcription factors STAT6 and KLF4, and thus establish MCPiP as the catalyst that connects the transcription factors, STAT6 and KLF4, to the biological processes they regulate.

Materials and Methods
Preparation and characterization of deubiquitinase mutant of MCPiP that retains RNase activity
Deletion mutants for the four potential ubiquitin-interacting domains were prepared, and the mutant proteins were expressed in HEK cells and purified and assayed for deubiquitinase activity with a model substrate Ub-AFC and with high m.w. K63-linked polyubiquitin (Boston Biochem), as described (13). One of the four mutants that showed loss of deubiquitinase activity is designated Dub mutant. This mutant was also assayed for RNAse activity as per manufacturer’s instructions (Applied Biosystems). Anti-Dicer RNAse activity of MCPiP and Dub mutant was measured using a synthetic pre-miRNA-133a tagged with a fluorophore in the loop and a quencher in the stem (5′-rCrArG rCrCrC rUrArU rGrUrG rArUrU rGrCc6-FAMK rGrUrC cCrCcA rArCcA uCrCcA uGrGu rArGGr U6-BHQ-1/cCrCcA-3′) (IDT). Purified MCPiP (5 μg) was incubated with 50 pmol pre-miRNA-133a in buffer containing 50 mM HEPES (pH 7.5), 100 mM potassium acetate, 10 mM magnesium acetate, 10 mM DTT, and 10% glycerol in a final volume of 200 μl. Dicer activity was measured by the increase in fluorescence caused by release of the fluorophore from the loop. The Dub mutant retained full RNAse and anti-Dicer activities. Experiments were performed in triplicates.

Generation of mice with myeloid-specific MCPiP knockout
A bacterial artificial chromosome clone containing 223,095 bp of mouse chromosome 4, including the entire MCPiP gene, was used to subclone the full-length MCPiP gene into a minimal vector containing an origin of replication and an Ampicillin resistance gene. The Gene Bridges’ BAC subcloning kit by RED/ET recombination was used to subclone a 9-kb segment of MCPiP gene, according to the manufacturer’s protocol. The subcloned 9 kb containing exon 2–6 along with the intervening introns was used to introduce loxP sites at introns 2 and 4 of the MCPiP gene using Gene Bridges’ Quick and Easy Conditional Knockout Kit (LoxP/Cre) by RED/ET recombination, according to the manufacturer’s protocol. Plasmid DNA from the final clone was purified and sequence confirmed prior to producing a linear fragment of the construct by EcoRV digestion. The linearized DNA segment containing the MCPiP-loxP construct was electroporated into C57BL/6J embryonic stem (ES) cells, and selection was made with myc tagged by PCR-based screening and Southern blot analysis were used to confirm homozygous recombination. ES cells containing the MCPiP-loxP construct were injected into blastocysts from cisogen strain C57Bl6 Ty+c2J, and homozygous line for MCPiP-loxP allele was produced by breeding and genotyping with PCR. The macrophage-specific MCPiP knockout mice (myelo-KO) were generated by crossing MCPiP-loxP+ mice with Ly-5Cre mice (The Jackson Laboratory), and LoxP+/-, Cre+ (myelo-KO) mice were identified by PCR genotyping.

Generation of mice with myeloid targeted overexpression of MCPiP
Murine LysM promoter (5352 bp) from mouse chromosome 10 position 116724852 to 116719328 was fused to murine MCPiP-FLAG in a pBlue-script vector. A 7332-bp NotI-Xhol fragment containing the LysM promoter fused to MCPiP was purified by gel electrophoresis and microinjected into fertilized C57BL/6J mouse OVA at the MD Anderson Cancer Center (Houston, TX). Genotyping was carried out using PCR with specific primers in the LysM promoter region and the transgenic coding region. The transgenic-containing founders were bred with C57BL/6J mice to generate F1 transgenic mice; homozygous myelo-MCPiP mice were produced by interbreeding.

Murine peritoneal macrophage isolation and culture
Thioglycollate-elicited macrophages were plated in six-well plates at a density of 1×10^6 cells/well in DMEM containing 10% FCS and 1% penicillin-streptomycin and 1% glutamine. After 4-h incubation, non-adherent cells were removed with PBS, new culture medium was added to the wells, and cells were subjected to treatment after 48-h culture. The following strains of mice were used for these experiments: MCPiP mice homozygous for targeting myeloid-specific expression of MCPiP or myeloid-specific deletion of MCPiP on the C57BL background. C57BL and MCPiP-LoxP homozygous mice were used as wild-type (WT) controls.

Plasmid construction for generating deletion mutants of MCPiP
Constructs with in-frame deletions of the four putative ubiquitin-interacting motifs identified using bioinformatics were created within MCPiP. The deletions encompassed the following sequences: U1-nucleotide 1–63, U2-nucleotide 379–417, U3-nucleotide 681–717, and U4-nucleotide 1112–1156. The WT MCPiP subcloned into the pCMV-MAT-FLAG vector was used as PCR template, and FluUltra II Fusion HS DNA Polymerase (Agilent) was used for the PCR generating the deletion mutants. The deletions for the internal motifs were achieved using a PCR technique known as gene splicing by overlap extension (24). The final PCR product was cloned into the pCR-Blunt II-TOPO vector; after sequencing to confirm the integrity of the sequence and the in-frame deletion, it was subcloned into pCMV-MAT-FLAG vector digested with HindIII and XbaI. The N-terminal deletion mutant MCPiP U1 was constructed by deleting the first 21 amino acids. To be able to monitor the transfection efficiency, the mutated MCPiP constructs were subcloned into the EcoRI-BamHI sites of pEGFP-N1 containing an enhanced GFP (GenBank: U55762.1).

Cell treatment, transfection, and small interfering RNA knockdown
Macrophages were seeded at 1×10^6 cells/well in six-well plates and cultured in complete DMEM for 2 d. After removal of the nonadherent cells, the attached cells were treated with LPS (100 ng/ml; Sigma-Aldrich) or IL-4 (20 ng/ml; Cell Signaling) for the reported time. For cell transfection, cells were transfected with either 1 μg pEGFP/N1 vector or 1 μg pEGFP-MCPiP expression plasmid, or expression vectors for D141N mutant or DUB mutant for 48 h, as previously described (10). For small interfering RNA (siRNA) knockdown studies, the attached cells were preincubated with Opti-MEM 1 medium containing lipofectamine (Invitrogen)/siRNA mixture (final concentration 100 nM siRNA) for 24 h before any treatments. siRNA for MCPiP, KLF4, and STAT6 was purchased from Life Technologies Ambion. For inhibitor intervention experiments, the attached cells were preincubated with 1 μM Co3O4 nanoparticles, 100 μM tauroursodeoxycholate (TUDC; Sigma-Aldrich), or 50 μM LY294002 (Sigma-Aldrich) in complete DMEM for 6 h before IL-4 treatment. Experiments were repeated at least three times.

Determination of intracellular ROS
The presence of free radicals in the macrophages after different stimulation was determined using dihydrodorhamidine 123 (Invitrogen), as described previously (18). Fluorescence images were obtained with a Nikon fluorescence microscope and NIS elements software (Nikon). In all cases, at least six different fields covering at least 200 cells were examined for quantifying the data.

Dual luciferase reporter assay
Macrophages were seeded at a concentration of 1×10^6 cells/well in six-well plates and cotransfected with 2 μg KLF4 expression vector (Addgene) and vector containing 1.5-kb MCPiP promoter fused to a luciferase gene in a 1:1 ratio using the Lipofectamine Transfection Reagent (Invitrogen) in FBS-free cell culture media. After 24-h exposure to the transfection mixture, the media was replaced with complete medium for 24 h, and then the cells were washed with Dulbecco’s PBS and lysed with 1× passive lysis buffer from Promega, and luciferase activity was measured by using the Dual Luciferase Reporter assay kit (Promega), according to the
manufacturer’s protocol. NF-κB activity was determined using the NF-κB Reporter kit (BPS Bioscience). Macrophages were seeded at 1 × 10^6 cells/well in six-well plates and cultured in complete DMEM for 2 d. After removal of the nonadherent cells, the attached cells were transfected with NF-κB reporter and negative control reporter for 24 h following the kit protocol. The cells were then incubated with Opti-MEM I medium containing Lipofectamine (Invitrogen)/MCPIP siRNA mixture (final concentration 100 nM siRNA) for 24 h, after which the cells were treated with LPS (100 ng/ml) for 6 h. After LPS treatment, the cells were lysed, according to the manufacturer’s instructions, and dual luciferase assay was performed using Dual-Glo Luciferase Assay System (Promega). NF-κB activity is reported as a ratio of firefly luminescence to Renilla luminescence. Experiments were performed in triplicates.

Quantitative real-time PCR

Total RNA was extracted from cell cultures using the Exiqon Micurry RNA isolation kit (Exiqon), according to the manufacturer’s instructions. cDNA was generated using a high-capacity cDNA reverse transcription kit (Applied Biosystems). Quantitative real-time PCR (qRT-PCR) was performed with 7500 Fast real-time PCR detection system (Applied Biosystems) using the SYBR Green master mix (Life Technologies). mRNA levels for M1 markers (TNF-α, IL-1β, IL-6, and inducible NO synthase [iNOS]), M2 markers (Arg1, MRC1, and FIZZ1), ER stress markers (GRP78, IRE-1), and autophagy marker (Beclin-1) were determined relative to the housekeeping gene β-actin, and fold differences within each group were calculated. Primers for gene expression assays were synthesized by Integrated DNA Technology (Table I). The same cDNA was used to measure the levels of mri155, 125, 223, and 146. Primers for miRs were purchased from Exiqon.

Immunoblot analysis

Macrophages were lysed with cell lysis buffer (Sigma-Aldrich), and the cell lysate was collected. For p47phox estimation, the cell lysate was centrifuged at 600 g for 10 min at 4°C to remove unbroken cells and nuclei. The supernatant was then ultra centrifuged at 100,000 g for 1 h at 4°C to isolate the membrane fraction. Protein was estimated by Bradford’s reagent, and equal amounts of protein sample from each experimental condition were subjected to immunoblot analysis using the following primary Abs: goat poly anti-MCP1 (1:500), rabbit anti-IRE-1 (1:500), rabbit anti-PPARγ (1:100), rabbit anti-ARG1 (1:200), and rabbit anti-LC3II (1:500), rabbit anti-C/EBPα (1:100), rabbit anti-FIZZ1 (1:500), monoclonal anti-p47phox (1:200), and anti-GAPDH Abs served as loading controls. Specific bands were quantified by densitometry using analytic software (Image J) and expressed as a ratio over loading controls.

Autophagosome measurement

The presence of autophagic vacuoles was determined using cyto-ID autophagic detection kit (Enzo Life Sciences) using the manufacturers' protocol. Fluorescence images were obtained with a Nikon fluorescence microscope, and fluorescence intensity was measured using NIS elements software (Nikon). In all cases, at least six different fields covering at least 200 cells were examined for quantifying the data.

Zymosan phagocytosis assay

Zymosan phagocytosis assay was done using pHrodo Red Zymosan Bioparticles (Life Technologies), according to the manufacturer’s instructions. Macrophages were plated at a density of 1 × 10^6 cells/well in 24-well plates in a final volume of 1 ml. After 24 h, fluorescently labeled (red) zymosan A (Life Technologies) was added at an approximate ratio of 1:5 (macrophage/zymosan). At the reported time points, cells were washed three times with PBS to remove free particles, and the amount of fluorescent intensity was analyzed by a fluorescence microscope, with three random fields per well (n = 3 wells per sample).

Animal protocol

The experiments with mice were approved by the animal care and use committee of the University of Central Florida and were in accordance with the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health.

Statistical analysis

All values are presented as mean ± SD. Significant differences were determined by two-way ANOVA for multiple comparisons. A p value < 0.05 is considered significant.

Results

To test whether IL-4–induced M2 polarization is mediated via MCP1, we tested whether IL-4 treatment of murine macrophages induces MCP1. The results showed that IL-4 treatment caused induction of MCP1 at both transcript and protein levels (Fig. 1A, 1B). If IL-4–induced expression of the M2 markers is mediated

### Table I. List of primers used for qRT-PCR

<table>
<thead>
<tr>
<th>Primers</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
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<tbody>
<tr>
<td>FIZZ1</td>
<td>Forward</td>
<td>5′-CCATTCGAGCTACTATCCGCC-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′-ACCCGCACTCCATATCC-3′</td>
</tr>
<tr>
<td>YM1</td>
<td>Forward</td>
<td>5′-AGAGGAGTGTACCTACAC-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′-CCTCTGCTGATGGCTGTAAGTG-3′</td>
</tr>
<tr>
<td>MRC1</td>
<td>Forward</td>
<td>5′-CCTCGTTTACGATGACCGC-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′-CGAGATTTCCTGGCTATTACGG-3′</td>
</tr>
<tr>
<td>Arg1</td>
<td>Forward</td>
<td>5′-TGCTGGGACAGAAGAC-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′-GGCTAGATGTGTGGCTTT-3′</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward</td>
<td>5′-GAGGATGAGCTTCCAGCATC-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′-CAGGTAAAAGACGCGCTTG-3′</td>
</tr>
<tr>
<td>Beclin-1</td>
<td>Forward</td>
<td>5′-ATGAGAGGCTGATGACCTGC-3′</td>
</tr>
<tr>
<td>GRP78</td>
<td>Forward</td>
<td>5′-GCCCAAGTGTCGTTCTACGGT-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′-ATCCAAGGTCACACACCTCGTGA-3′</td>
</tr>
<tr>
<td>IRE-1</td>
<td>Forward</td>
<td>5′-TGATCCCTCCCTTCATATTGCT-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′-CACCACCTGAGGCTGTTTC-3′</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Forward</td>
<td>5′-GGAGAATTTCTGGGATTCAGCT-3′</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Forward</td>
<td>5′-GAAGATTTCTCAAGAGAAGACG-3′</td>
</tr>
<tr>
<td>IL-6</td>
<td>Forward</td>
<td>5′-ATCCGAACTGAGCTGTTAC-3′</td>
</tr>
<tr>
<td>β-actin</td>
<td>Forward</td>
<td>5′-ATGGTTTACGACCTTCACAAC-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′-CAGATGACCTTCATGAGG-3′</td>
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via MCPIP, knockdown of MCPIP should inhibit the IL-4–induced M2 marker expression. In fact, MCPIP-specific siRNA, which knocked down MCPIP expression (Fig. 1C), inhibited the IL-4–induced expression of M2 markers Arg1, YM1/chitinase-like lectin, and FIZZ1/Relm-α (Fig. 1D–H), whereas nontarget siRNA did not affect the induction of these markers at both transcript and protein levels. If IL-4–induced M2 polarization is mediated via induction of MCPIP, forced expression of MCPIP should induce M2 polarization of murine macrophages without IL-4 treatment. To test for this possibility, we transfected murine macrophages with MCPIP expression vector without IL-4 addition. The results showed that MCPIP expression alone induced expression of M2 markers (Arg1, FIZZ1) at both transcript and protein levels (Fig. 2A–D).

Because IL-4 is known to function via induction of STAT6 that is known to induce KLF4 to mediate M2 polarization (9), we tested whether forced expression of KLF4 could induce MCPIP. Transfection of murine macrophages with KLF4 expression vector induced MCPIP at both transcript and protein levels (Fig. 3A, 3D). To further test whether the multiple KLF4 binding sites in MCPIP promoter were responsible for KLF4 induction of MCPIP, we fused a 1.0-kb 5′-flanking region of MCPIP gene, containing the multiple KLF4 sites, to luciferase gene and tested whether KLF4 expression would drive luciferase expression. Murine macrophages transfected with the luciferase fusion construct showed luciferase expression only upon cotransfection with KLF4 expression vector (Fig. 3F). These results clearly showed that IL-4–induced MCPIP expression was mediated via KLF4. IL-4 induction of M2 polarization of macrophages was reported to be mediated by inhibition of M1 polarization mediated via NF-κB activation, and KLF4 was reported to mediate this inhibition (9).

Because MCPIP is known to suppress NF-κB activation (12, 13), we tested whether the KLF4-mediated NF-κB inhibition is implemented via MIPCIP. LPS-induced expression of NF-κB target

FIGURE 1. MCPIP is required for IL-4–induced M2 macrophage polarization. The peritoneal macrophages isolated from C57BL/6 mice were treated with IL-4 (20 ng/ml) at the indicated time points. Expression of MCPIP was assayed by qRT-PCR and immunoblots. *p < 0.05 versus untreated cells (A and B). Mouse macrophages were transfected with nontargeted siRNA or siRNA against MCPIP for 24 h, and then treated with 20 ng/ml IL-4 for 4 h. After 4 h, RNA and protein were isolated. Knockdown of MCPIP by treatment with siRNA against MCPIP inhibited IL-4–induced expression of MCPIP, assayed by qRT-PCR (C). Knockdown of MCPIP by siRNA against MCPIP inhibited IL-4–induced expression of M2 markers (Arg1, YM1, FIZZ1), assayed by qRT-PCR (D–F) and immunoblot (G and H). Each experiment was repeated three times. *p < 0.05 versus controls. #p < 0.05 versus nontarget (NT) siRNA.
genes, iNOS, IL-1β, TNF-α, and IL-6 was suppressed by forced expression of KLF4, and this suppression was reversed by knocking down MCPIP with specific siRNA (Fig. 3H). To further test whether KLF4-mediated suppression of M1 polarization is mediated through suppression of NF-κB activation via MCPIP, we used luciferase reporter assay. LPS induction of luciferase driven by NF-κB was suppressed by forced expression of KLF4 (Fig. 3G). If this suppression is mediated via MCPIP, knockdown of MCPIP with specific siRNA should reverse the suppression by KLF4 expression. In fact, luciferase activity was recovered by treatment with siRNA specific for MCPIP, but not with non-targeted siRNA (Fig. 3G). These results provide direct evidence for the involvement of MCPIP in the KLF4-induced suppression of NF-κB activation.

ROS production, ER stress, and autophagy have been reported to be involved in IL-4–induced M2 polarization (20–22). MCPIP is known to induce oxidative stress, ER stress, and autophagy in several cell types (17–19). We tested whether MCPIP mediates the IL-4–induced generation of ROS, ER stress, and autophagy in murine macrophages. Knockdown of MCPIP with specific siRNA should reverse the suppression by KLF4 expression. In fact, luciferase activity was recovered by treatment with siRNA specific for MCPIP, but not with non-targeted siRNA (Fig. 3G). These results provide direct evidence for the involvement of MCPIP in the KLF4-induced suppression of NF-κB activation.

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Even though ROS production, ER stress, and autophagy induced by IL-4 treatment of murine macrophages were reported to be involved in M2 polarization (20–22), the relationships among these processes have not been investigated. We used selective inhibitors of these processes to test the sequence of MCPIP-mediated processes involved in M2 polarization. Inhibition of ROS production with CeO2 nanoparticles inhibited ER stress formation as measured by GRP78 and IRE-1 expression levels (Fig. 5A, Supplemental Fig. 1A, B). This inhibition of ROS production also blocked autophagy, as measured by Beclin-1 level and autophagosome staining, and induction of M2 markers, Arg1 and FIZZ1, at both transcript and protein levels (Fig. 5B, C, D, Supplemental Fig. 1C, D, E, F).

Inhibition of ER stress with TUDC blocked autophagy as measured by Beclin-1 expression, LC3II:LC3I ratio, and autophagosome staining (Fig. 5B, Supplemental Fig. 1A, D). This inhibition of ROS production also blocked autophagy, as measured by Beclin-1 level and autophagosome staining, and induction of M2 markers, Arg1 and FIZZ1, at both transcript and protein levels (Fig. 5C, D, Supplemental Fig. 1C, D).
protein levels, caused by IL-4 treatment (Fig. 5F, 5G, Supplemental Fig. 2D, 2E). Inhibition of autophagy with LY294002 inhibited induction of expression of M2 markers, caused by IL-4 treatment (Fig. 5H, 5I, Supplemental Fig. 2F, 2G).

How MCPIP might initiate the sequential process was examined by testing whether MCPIP induces p47phox, a critical component of NADPH oxidase responsible for ROS production. Transfection of murine macrophages with MCPIP expression plasmid induced p47phox (Fig. 5J), suggesting that induction of ROS production by MCPIP expression is mediated via p47phox. The level of p47phox protein also showed statistically significant elevation in both soluble and membrane fractions of the cell lysate (Fig. 5K, 5L).

MCPIP has deubiquitinase (13) and RNase activities, including anti-Dicer activity (12, 14, 15). The anti-Dicer RNase activity cleaves the loop from the pre-miR and thus depletes the substrate for Dicer, causing inhibition of miR synthesis (25). To determine whether either or both of these activities of MCPIP are involved in M2 polarization, we needed MCPIP mutants with only one of these activities. It has been shown that D141N mutant of MCPIP has lost RNase and anti-Dicer activities (14, 24), but retains its deubiquitinase activity against model substrate, high m.w. polyubiquitin, and in vitro ubiquitinated HIF1α (23). Because no mutant was available that has lost only deubiquitinase activity with retention of RNase activity, we attempted to generate such a mutant. We deleted the four potential ubiquitin-interacting domains in MCPIP, and all four mutant proteins were assayed for deubiquitinase activity. Only one of them was found to have lost deubiquitinase activity against both a model substrate (Fig. 6A) and high m.w. polyubiquitin (Fig. 6B). This mutant showed full retention of RNase activity as measured with a commercially available general RNase activity kit (Fig. 6C). Anti-Dicer activity was measured by a novel assay that we designed in which we used synthetic pre-miR135a tagged with a fluorophore in the loop and a quencher in the stem. Anti-Dicer activity released the fluorophore from the loop, causing increase in fluorescence. With this assay, this mutant showed full retention of anti-Dicer activity (Fig. 6D).

FIGURE 3. IL-4–induced MCPIP induction is via STAT6/KLF4, and KLF4-induced NF-κB inhibition is mediated via MCPIP. The peritoneal macrophages isolated from C57BL/6 mice were pretreated with siRNA against KLF4 and STAT6 for 24 h, and then treated with IL-4 (20 ng/ml) for 4 h. Expression of MCPIP and M2 markers (Arg1, FIZZ1) was determined by qRT-PCR (A–C). Transfection with KLF4 expression plasmid induced MCPIP expression in murine macrophages, as determined by qRT-PCR (D) and Western blot (E). Murine macrophages were transfected with MCPIP promoter-luciferase construct with or without cotransfection with KLF4 expression vector for 24 h, and luciferase activity was measured in the lysate. Expression of KLF4 enhanced MCPIP-luciferase reporter activity (F). Mouse macrophages were pretreated with siRNA against MCPIP for 24 h and then treated with 100 ng/ml LPS for 6 h. KLF4 suppression of LPS-induced NF-κB activity was prevented by siRNA knockdown of MCPIP, as measured by the NF-κB reporter kit (G). KLF4 suppression of LPS-induced NF-κB target genes (iNOS, IL-1β, TNF-α, and IL-6), assayed by qRT-PCR, was prevented by siRNA knockdown of MCPIP (H). Experiments were repeated three times. *p < 0.05 versus untreated control cells. #p < 0.05 versus nontargeted (NT) siRNA.
We tested the RNase mutant and Dub mutant for their activity to induce M2 polarization by measuring induction of M2 marker gene expression after transfection of murine macrophages with expression vector for WT or the two mutants of MCPIP. The results showed that both Dub mutant and RNase mutant were very much less effective in inducing the expression of M2 markers, Arg1 and FIZZ1, at both transcript and protein levels than the WT MCPIP, indicating that both catalytic activities of MCPIP were necessary for the induction of M2 polarization (Fig. 2A–D).

Because ROS production was reported to be critical for IL-4–induced M2 polarization (20), we tested whether production of ROS was affected by loss of either catalytic activity of MCPIP. When murine macrophages were transfected with expression vectors for WT MCPIP and the two mutants, it was found that ROS production was inhibited by loss of either of the two activities of MCPIP (Fig. 2E). We tested whether loss of either catalytic activity of MCPIP would affect induction of ER stress or autophagy. Induction of ER stress as indicated by expression of GRP78 was inhibited by loss of either deubiquitinase or RNase activity of MCPIP, but IRE-1 expression was inhibited by its mutations only at the transcript level (Fig. 2F, 2G, Supplemental Fig. 3A, 3B). Induction of autophagy, as indicated by Beclin-1 expression, was severely inhibited by loss of RNase activity, but not by loss of deubiquitinase activity (Fig. 2H, Supplemental Fig. 3C, 3D). LC3II protein levels induced by MCPIP were significantly lower with both mutants (Fig. 2I).

PPARγ and C/EBPβ, two transcription factors known to promote M2 polarization (26–28), are known to be induced by IL-4 treatment of murine macrophages (27, 28). Knockdown of MCPIP with specific siRNA, but not nontargeted siRNA, inhibited induction of PPARγ and C/EBPβ by IL-4 treatment of murine macrophages (Fig. 6E, 6F). We tested whether either catalytic activity of MCPIP is involved in the induction of these M2-associated transcription factors. PPARγ induction was severely inhibited by loss of RNase activity and less severely inhibited by loss of deubiquitinase activity (Fig. 6G). Induction of C/EBPβ...
was severely inhibited by loss of RNase activity of MCPIP, but was not significantly affected by loss of deubiquitinase activity.

Because anti-Dicer RNase activity would be involved in miR synthesis that might be associated with macrophage polarization, we tested whether the loss of anti-Dicer RNase activity of MCPIP affects the production of miR known to be involved in macrophage polarization. qRT-PCR measurements of miR levels showed that expression of M2-associated miRs 223 and 146 was enhanced by MCPIP expression, but this induction was suppressed by loss of RNase activity of MCPIP (Fig. 6H). In contrast, production of M1-associated miRs, 155 and 125, was dramatically suppressed by MCPIP when compared with the RNase mutant of MCPIP (Fig. 6I). These results suggest that anti-Dicer activity of MCPIP participates in the regulation of M2 polarization via control of miR production.

To determine the effect of MCPIP in M2 polarization in vivo, we generated mice with myeloid-targeted expression of MCPIP (myelo-MCPIP). LysM promoter was used to drive MCPIP expression.
specifically in differentiated macrophages (29). The peritoneal macrophages isolated from the myelo-MCPIP mice clearly showed elevated MCPIP expression at both the transcript level and protein level when compared with the very low levels found in the macrophages from the WT mice (Supplemental Fig. 4E, 4F).

Peritoneal macrophages from myelo-MCPIP mice showed suppressed expression of M1 marker genes upon treatment with LPS (Fig. 7G). Thus, 2- and 4-h treatment of the macrophages from the transgenic mice with 100 ng/ml LPS showed suppressed expression of iNOS, TNF-α, and IL-1β (Fig. 7G–I). In contrast, peritoneal macrophages from myelo-MCPIP mice displayed significant induction of M2 marker genes, Arg1, YM1, and FIZZ1 (Fig. 7J–L). These results show that MCPIP plays a critical role in macrophage polarization in vivo (Fig. 8).

Discussion
The remarkable plasticity of macrophages allows them to respond to environmental signals. Infection, injury, and inflammatory stimuli cause activation of macrophages to M1 state that allows them to defend the host against invading pathogens or other harmful agents. However, sustained inflammatory condition, represented by M1-activated macrophages, can damage the host. To protect the host from such damage, the macrophages undergo M2 polarization that is anti-inflammatory. This differentiation is triggered by clues generated by the inflammatory state. MCP-1 and other inflammatory chemokines and cytokines are produced by macrophages via NF-κB activation that occurs during macrophage M1 activation. MCP-1 interaction with its receptor CCR2 causes signal transduction events that result in the induction of a zinc-finger protein called MCPIP (10). MCPIP is also induced by other inflammatory signals such as IL-1β, TNF-α, and IL-6 (11, 12). Inflammatory signals also trigger formation of agents that lead to the eventual production of anti-inflammatory components that mediate resolution of inflammation to protect the host from damage from sustained/excessive inflammation. Our results strongly suggest that MCPIP is a critical inflammation-induced agent that promotes the polarization of macrophages into anti-inflammatory M2 state using its multiple catalytic activities.
It was reported that IL-4–induced M2 polarization involved STAT6 and KLF4 that induce each other and function cooperatively to induce M2 polarization (9). It was also pointed out that KLF4-mediated induction of PPARγ probably contributes to the inhibition of M1 polarization. IL-4 binding to its receptor causes signal transduction that results in tyrosine phosphorylation in STAT6, leading to dimerization and nuclear entry to cause transcriptional activation of genes, including PPARγ (27). PPARγ regulates fatty acid metabolism, promoting the aerobic respiration that occurs in M2 macrophages. Our results indicate that IL-4–induced PPARγ induction is mediated by MCPIP. The role of MCPIP in implementing the functions of the transcription factors, STAT6 and KLF4, is mediated via its deubiquitinase activity and RNase activity. Results presented in this work indicate that both catalytic activities are involved in mediating M2 polarization. IL-4 induction of M2 polarization mediated by KLF4 inhibits M1 polarization and promotes M2 polarization. Deubiquitinase activity of MCPIP can prevent NF-κB activation via removal of ubiquitins from the components like TNFR-associated factor 6 and high m.w. polyubiquitins involved in IκB kinase activation required for NF-κB activation (13). Because ubiquitination state regulates the stability and function of many proteins, it is yet to be determined whether other components involved in inhibition of M1 and promotion of M2 polarization are regulated by the deubiquitinase activity of MCPIP. Recently, immunopurifications and mass spectrometric analysis revealed that >20 proteins may be directly or indirectly associated with ubiquitin in IFN-γ/TLR-stimulated RAW cells, implicating the role of ubiquitin modification involved in immune responses (30).

The role of RNase activity of MCPIP includes previously reported inhibition of synthesis of inflammatory cytokines such as IL-1β and IL-6 by causing degradation of their mRNA (12, 14, 15). The anti-Dicer activity would inhibit miR synthesis by its cleavage of the loop from pre-miR. The role of miR in macrophage polarization is only beginning to be elucidated. Using microarrays, changes in miR levels associated with macrophage polarization in human (31) and murine (32) macrophages were examined. The number of miRs that showed significant changes ranged from 109 in murine to 249 in human macrophages, with a dozen or so showing major changes in expression. Obviously, the regulation of processes involved in macrophage polarization by miR remains poorly understood. However, there are examples that point to possible ways by which the anti-Dicer activity of MCPIP would regulate macrophage polarization. Suppression of production of miRs that negatively regulate M2 polarization may promote M2 polarization. miR155 is upregulated in M1 macrophages, where it promotes production of inflammatory MCP-1 and TNF-α (33, 34). It also reduces expression of some M2 markers such as Arg1. We provide evidence that the suppression of synthesis of miR155 and miR125 by MCPIP is probably involved in the induction of M2 polarization. The MCPIP mutant, which has lost anti-Dicer activity, does not suppress the production of these miRs that are known to inhibit M2 polarization and thus lost the ability to induce M2 polarization in murine macrophages. Expression of M2-associated miRs (miR223 and miR146) was enhanced by MCPIP.
is required for M2 polarization, is shown to be mediated via NF-κB activation and promotes M2 polarization by sequentially inducing ROS production, ER stress, and autophagy required for M2 polarization.

expression, but was downregulated by the RNase mutant. These results suggest that the anti-Dicer activity of MCPIP is involved in M2 polarization. How the miRs regulate macrophage polarization is yet to be fully elucidated.

CREB/CEBP cascade causes transcriptional activation of M2 macrophage markers Arg1, IL-10, and MRC1 (28). Arg1 gene promoter has STAT6 and C/EBP sites that are involved in M2 polarization. C/EBP synergizes STAT factors on anti-inflammatory promoters. The M2 program was reported to be specifically sensitive to C/EBP levels (28). An E3 ligase, Nrdp1, was reported to K-63 ubiquitinate C/EBPβ and activate it to enhance transcription of Arg1 in IL-4–polarized macrophages (35). In the macrophages of transgenic mice expressing Nrdp1, not only Arg1, but also other M2 markers such as YM1, FIZZ1, and IL-10 were upregulated. Nrdp1 expression promoted M2 polarization also by inhibiting LPS induction of iNOS, TNF-α, IL-1β, and IL-6 in murine macrophages. Results presented in this work show that IL-4 induces C/EBPβ via MCPIP.

IL-4–induced M2 polarization was reported to require ROS production (20–22). Tumor-associated macrophages that are mostly M2 type are involved in tumorigenesis owing to their proangiogenic functions. Antioxidants that prevented the formation of ROS were found to inhibit M2 polarization and markedly suppressed tumorigenesis (36). We have previously reported that MCPIP induces oxidative stress in other cellular contexts, at least in part, via induction of p47phox (17, 18). In this work, we demonstrate that ROS production involved in IL-4–induced M2 polarization is mediated via MCPIP, as indicated by the finding that knockdown of MCPIP inhibited IL-4–induced ROS production.

It has been reported that ER stress controls M2 macrophage differentiation (22, 37). Alternative activation of macrophages from diabetic human patients, induced by IL-4, manifested unfolded protein response (37). Inhibition of ER stress with chemical chaperone, phenylbutyric acid, prevented the M2 gene expression pattern and showed low IL-10 production. Furthermore, induction of ER stress by thapsigargin treatment of human macrophages induced M2 marker expression (37). These results indicated that inhibition of ER stress inhibited M2 marker expression. MCPIP expression has been reported to induce ER stress in some cell types (17–19). In the present case, IL-4–induced ER stress, which is required for M2 polarization, is shown to be mediated via MCPIP, as indicated by the finding that knockdown of MCPIP with specific siRNA inhibited IL-4–induced ER stress and M2 polarization.

Recently, it was reported that cathepsin S–mediated autophagic flux is involved in promoting M2 polarization in tumor-associated macrophages (36). Cathepsin S–deficient macrophages failed to manifest autophagic flux and M2 polarization in a tumor environment (36). Treatment with an inhibitor of autophagy, chloroquine, also inhibited M2 polarization (21). The present results that IL-4–induced M2 polarization involves autophagy are consistent with previous reports. Because knockdown of MCPIP with specific siRNA inhibited IL-4–induced autophagy and M2 polarization, it is highly likely that IL-4–induced M2 polarization in murine macrophages involves MCPIP-mediated autophagy. Autophagy would allow degradation of proteins that represent M1 state to provide amino acids for the synthesis of proteins involved in M2 state. The role of autophagy in differentiation is to provide amino acids for the synthesis of proteins needed for the differentiated state.

Even though ROS, ER stress, and autophagy were reported to be involved in IL-4–induced M2 polarization, if and how such biological processes cooperatively function in M2 polarization have not been examined. The present results indicate that IL-4–induced M2 polarization involves ROS production that causes ER stress that leads to autophagy involved in the differentiation process involved in M2 polarization. Thus, inhibition of each step leads to inhibition of subsequent steps in the sequential process. CeO2 nanoparticles that inhibit oxidative stress inhibit ER stress and autophagy and M2 marker expression. Inhibition of ER stress with a chemical chaperon (TUDC), which is known to selectively inhibit ER stress (38), inhibits autophagy and M2 marker expression. Inhibition of autophagy with a selective chemical inhibitor, LY294002, inhibits M2 marker expression. These results are consistent with previous reports about the involvement of sequential induction of oxidative stress, ER stress, and autophagy in adipogenesis, angiogenesis, and osteoclastogenesis induced by MCPIP (17–19).

Results presented in this work demonstrate that mediation of M2 polarization by MCPIP involves both its deubiquitinase and RNase activities. Thus, MCPIP mutants that lost either one of the two catalytic activities failed to induce ROS production, ER stress, autophagy, and expression of M2 markers. Results presented in this work demonstrate that IL-4–induced M2 polarization is mediated via KLF4-mediated induction of MCPIP that implements M2 polarization via exerting its posttranscriptional regulation by its RNase activity and its posttranslational level regulation by its deubiquitinase activities. It is demonstrated that MCPIP mediates ROS production that causes ER stress that leads to autophagy involved in M2 polarization. The central role played by MCPIP in M2 polarization is summarized in Fig. 8. Transcription factors implement their biological function via the catalytic property of proteins that function as a link between the transcription factors and the biological processes they regulate. The results presented in this study show that the dual catalytic activities of MCPIP implement a function of the two transcription factors STAT6 and KLF4. Even though RNase activity of MCPIP has been well documented by several laboratories (14, 15), whether MCPIP has deubiquitinase activity has been questioned (39). With a new MCPIP mutant that lost deubiquitinase activity, but retains full RNase activity, we demonstrate that the deubiquitinase activity of MCPIP plays an important biological function. The substrates for the two catalytic activities that play critical roles in the biological processes involved in M2 polarization remain to be identified.

Macrophage polarization plays important roles in some major diseases. For example, obesity and type 2 diabetes, which are major
health problems in the world, involve macrophages that infiltrate the adipose tissue (26). KLF4, which mediates MCPIP induction, is expressed at a reduced level in obese people (9). Furthermore, this inducer of MCPIP is expressed at a lower level in visceral versus s.c. fat in obese subjects. Visceral fat is associated with insulin resistance. This difference in KLF4 levels probably would result in lower MCPIP levels, and consequently, macrophages associated with visceral fat would be more M1 than M2. M1 macrophages are well known to be associated with insulin resistance (26, 40). Macrophage polarization is also relevant to cardiovascular diseases. For example, M1/M2 plays a role in atherosclerosis. M1 phenotype is more prevalent in rupture-prone places in the plaques, such as plaque shoulder (8, 41). M1 macrophages are exclusively found in plaques of symptomatic patients and at higher levels in unstable plaques, whereas M2 macrophages are found to be higher in stable plaques in both symptomatic and asymptomatic patients. Thus, M1/M2 ratio and their location within the atherosclerotic lesion can be critical for plaque stability.

The tumor-associated macrophages tend to be of M2 phenotype (42, 43). These macrophages promote angiogenesis and thus promote tumor growth. M2 macrophages also promote wound healing presumably by promoting angiogenesis involved in effective wound healing, cardiovascular disease, and cancer (6, 44).

Absence of KLF4 in macrophages caused increased phagocytic activity, as absence of KLF4 favored M1 polarization (9). This effect of KLF4 is probably mediated via its ability to induce MCPIP demonstrated in this work. The present results show that absence of MCPIP in macrophages favored M1 phenotype with higher bactericidal activity. Thus, the critical role of MCPIP in M2 polarization is relevant to major health issues such as obesity and insulin resistance in type 2 diabetes, control of bacterial infection, angiogenesis involved in wound healing, and cancer. Thus, MCPIP could be a suitable target for intervention in such human health problems.

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Disclosures

The authors have no financial conflicts of interest.

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