

lncRNA-MM2P downregulates the production of pro-inflammatory cytokines in acute gouty arthritis

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Abstract. Acute gouty arthritis (AGA) is characterized by the accumulation of pro-inflammatory cytokines, which are immunological responses to monosodium urate (MSU) crystals. It has been demonstrated that long non-coding RNA (lncRNA)-MM2P is a novel regulator of M2 polarization of macrophages. The aim of the present study was to investigate whether lncRNA-MM2P regulates the MSU-induced inflammatory process. In cell models of RAW 264.7 and THP-1-derived macrophages, decreased expression of lncRNA-MM2P was observed in lipopolysaccharide- and MSU-treated macrophages, which was accompanied with obvious inflammatory responses. Using small interfering RNA to knockdown lncRNA-MM2P led to the upregulation of MSU-mediated inflammatory responses, both in RAW 264.7 and THP-1-derived macrophages. In conclusion, lncRNA-MM2P could be an important regulator of MSU-induced inflammation, and therefore could be involved in the development of AGA.

Introduction

Acute gouty arthritis (AGA) is a common and severe form of inflammatory arthritis (1). Epidemiological evidence demonstrates that the prevalence of gout is rapidly increasing (2). AGA is initiated by the deposition of monosodium urate (MSU) crystals in the articular joints and bursal tissues (3-5). Exploring the underlying mechanisms of MSU-induced inflammation is important in the treatment of AGA (6).

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Macrophages play an important role in the pathogenesis of inflammation. Macrophages are usually classified into classical subtype (M1) and alternative subtype (M2), according to their pro-inflammatory and anti-inflammatory functions, respectively (7). Physiologically, the balance of M1/M2 macrophage polarization is well maintained. However, it has been demonstrated that an imbalance in M1/M2 polarization is crucial in the development of MSU-induced inflammation (8). Molecular mechanisms of M1 polarization in AGA includes the sirtuin 1/PI3K/Akt/STAT6 pathway, cyclooxygenase-2 and secreted frizzled-related protein 2 (9-11), while M2 polarization in AGA is relatively less understood.

Recently, long non-coding RNA (lncRNA)-MM2P has been identified as a novel modulator of M1/M2 polarization and a promoter of tumorigenesis. A microarray-based profiling assay demonstrated that lncRNA-MM2P is the only lncRNA upregulated during M2 polarization and downregulated in M1 macrophages (12). Therefore, the present study aimed to investigate whether lncRNA-MM2P is an important modulator of MSU-induced inflammation in cell models of RAW 264.7 and THP-1 monocytes.

Materials and methods

MSU crystal synthesis. Briefly, 100 mg of uric acid ($\geq 99\%$, crystalline; cat. no. U2625; Sigma-Aldrich; Merck KGaA) was dissolved, heated at 60°C and blended in 20 ml of distilled water with 60 μ l of 10 mol/L⁻¹ NaOH, adjusted to pH 7.2-7.4 with HCl (1 mol/L⁻¹) at 60°C. The solution was kept overnight under constant shaking at 60°C and then kept at room temperature for 5 days. After 5 days, the mixture was transferred to a 15 ml tube and stored at 4°C for 4 days. Needle-like crystals were recovered and suspended using a vortex overnight. The crystals were collected after washing twice with 100% ethanol and once with acetone, followed by centrifugation at 990 x g for 2 min at 4°C. The MSU crystals were suspended in sterile endotoxin-free phosphate-buffered saline (PBS). The crystals obtained were preserved at -20°C after evaporation by heating at 180-200°C (13,14).

Cell culture and treatment. RAW 264.7 cells (Mus musculus, mouse, ATCC® TIB-71™) and THP-1 cells were obtained from

American Type Culture Collection. Cells were maintained in DMEM (Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Sigma-Aldrich; Merck KGaA), 100 U/ml penicillin and 100 µg/ml streptomycin in a humidified atmosphere of 5% CO₂ at 37°C. Cells were allowed to grow until they reached 90-95% confluence, following which they were washed with PBS and the culture medium was replaced. RAW 264.7 cells attaining a concentration of 5x10⁴ cells/ml were activated by incubation in medium containing MSU crystals (0, 10, 50, 250 µg/ml) for 24 h at 37°C. These concentrations of MSU were chosen according to previous studies (15-17). Cells were also stimulated with 1 mg/ml Escherichia coli-derived lipopolysaccharide (LPS; serotype 0111:B4; cat. no. L2630; Sigma-Aldrich; Merck KGaA) for 0, 6, 12 and 24 h at 37°C. THP-1 monocytes were suspended in complete culture medium and seeded in 24-well culture plates (2x10⁵ cells/ml/well). The cells were then treated with 100 ng/ml phorbol myristate acetate for 24 h at 37°C to obtain THP-1-derived macrophages. THP-1-derived macrophages in DMEM were added to different concentrations of MSU crystals (0, 10, 50, 250 µg/ml) for 24 h at 37°C. After transfection with lncRNA-MM2P siRNA and scramble siRNA, cells (RAW 264.7 and THP-1-derived macrophages) were further treated with or without MSU crystals (250 µg/ml) for 24 h at 37°C. The harvested cells were collected for RNA and protein extraction. The supernatants were collected and stored at -80°C for cytokine detection by ELISA (13,14).

Plasmids and cell transfection. RAW 264.7 and THP-1 cells in the logarithmic growth phase were collected and seeded into six-well plates at a density of 5x10⁴ cells/well. Transfection was performed when the cell density reached 80% the following day. The small interfering RNA (siRNA) sequence duplexes were produced by Shanghai GenePharma Co., Ltd. The lncRNA-MM2P siRNA sequences were as follows: si-lncRNA-MM2P-1, 5'-CACGAAGACTGGAATGCAATT-3'; and si-lncRNA-MM2P-2, 5'-GGACCGAAGAGATTCGGAGAA-3'. The control (scramble siRNA) sequence was as follows: 5'-ATCCGCGCGATAGTACGTATT-3'. The transfection was performed using 50 nM siRNA and jetPRIME® (Polyplus-transfection SA), according to the manufacturer's protocols (12). lncRNA-MM2P was cloned into a pcDNA3.1 vector (Shanghai Integrated Biotech Solutions Co., Ltd.) and transfected into cells (0.5 µg/ml) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). An empty pcDNA3.1 vector was transfected into the cells as the control. The primer sequence for lncRNA-MM2P was as follows: Forward, 5'-TAGCTCCCACGAAGACTGGAAT-3' and reverse, 5'-CTATGCTCGTGATTTATAAAACGCAAGTC-3'. The subsequent experiments were performed following 48 h of transfection at 37°C.

Reverse transcription-quantitative (RT-q)PCR. Total RNA from RAW 264.7 and THP-1-derived macrophages was extracted from cultured cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. To quantitate lncRNA-MM2P, interleukin (IL)-1β, IL-8 and tumor necrosis factor α (TNFα) mRNA, cDNA was reverse transcribed from total RNA using the High-Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific, Inc.)

at 70°C for 10 min. Then, 1 µg cDNA was used with the primers shown below in a 20 µl reaction, using SYBR-Green as a marker for DNA content, provided in the SYBR-Green Master mix (Applied Biosystems; Thermo Fisher Scientific, Inc.). The primer sequences used for RT-qPCR were as follows: lncRNA-MM2P, forward: 5'-TAGCTCCCACGAAGACTGGAAT-3' and reverse: 5'-CTATGCTCGTGATTTATAAAACGCAAGTC-3'; IL-1β, forward: 5'-ACGATGCACCTGTACGATCA-3' and reverse: 5'-TCTTTCAACACGCAGGACAG-3'; IL-8, forward: 5'-GCATAAAGACATACTCCAAACC-3' and reverse: 5'-ACTTCTCCACAACCCTCTG-3'; TNFα, forward: 5'-CAGAGGGAAGAGTTCCTCCAG-3' and reverse: 5'-CCTTGGTCTGGTAGGAGACG-3'; GAPDH, forward: 5'-GGAAGGTGAAGGTCGGAGTCA-3' and reverse: 5'-GTCATTGATGGCAACAATATCCACT-3'. Amplification was performed in an ABI-Prism 7000 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) for a maximum of 40 cycles as follows: 94°C for 40 sec; then, 58°C for 40 sec and 72°C for 1 min. The expression levels were quantified using the 2^{-ΔΔCq} method (18). Reactions were performed in triplicate for statistical analysis, similar to our previous studies (12-14).

ELISA to measure cytokine levels. The generation of inflammatory gene products (IL-1β, IL-8 and TNFα) by RAW 264.7 cells was assayed using the following ELISA kits purchased from Abcam: Human IL-1β ELISA kit (cat. no. ab46052), human IL-8 ELISA kit (cat. no. ab46032) and human TNFα ELISA kit (cat. no. ab181421). Generation of inflammatory gene products (IL-1β, IL-8 and TNFα) by THP-1-derived macrophages was assayed using the following ELISA kits, mouse IL-1β ELISA kit (cat. no. ab197742; Abcam), mouse IL-8 ELISA kit (cat. no. MOFI01258; ELISA Genie) and mouse TNFα ELISA kit (cat. no. ab100747; Abcam). Cells (1.5x10⁶ cells/well) seeded in 6-well plates were pre-incubated with various concentrations of morin (Mingxiu Biotechnology Company; 100-300 µM) or colchicine (Mingxiu Biotechnology Company; 1 µM) for 24 h at 37°C, and cytokine production was stimulated by treating cells with MSU crystals (1 mg/ml) for 24 h at 37°C. Then, the culture supernatants were collected and used to measure levels of IL-1β, IL-8 and TNFα. Absorbance was determined at 450 nm using a microplate reader (BioTek Instruments, Inc.). The standard curves for respective cytokines were used to quantify the levels of IL-1β, IL-8 and TNFα released by the cells.

Statistical analysis. The data was presented as the mean ± SD for normally distributed variables, and medians (interquartile range) for non-normally distributed variables. An unpaired two-tailed Student's t-test was used to determine significance between controls and individual experimental groups. A one-way ANOVA followed by a Tukey's post hoc test was used for >3 groups. Analyses were performed using SPSS software, v22.0 (IBM Corp.). P<0.05 was considered to indicate a statistically significant difference.

Results

Treatment with LPS and MSU decreases the expression of lncRNA-MM2P in macrophages. RAW 264.7 and THP-1-derived macrophages were treated with LPS at four time

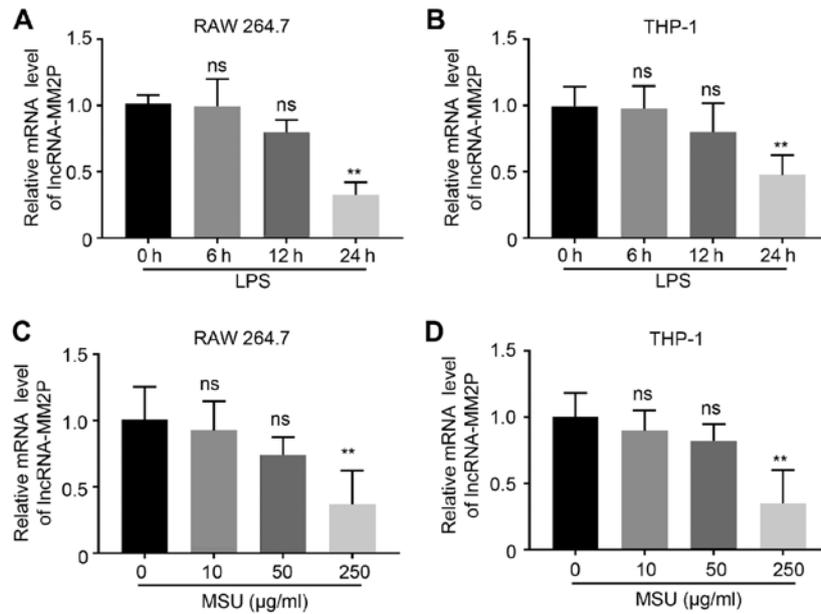


Figure 1. Effects of LPS and MSU treatment on the expression of lncRNA-MM2P in RAW 264.7 and THP-1 cells. The expression of lncRNA-MM2P in (A) RAW 264.7 and (B) THP-1 cells was reduced by treatment with LPS from 6 to 24 h. The expression of lncRNA-MM2P in (C) RAW 264.7 and (D) THP-1 cells was reduced by treatment with MSU from 6 to 24 h. **P<0.01 vs. 0 h. LPS, lipopolysaccharide; MSU, monosodium urate; lncRNA, long non-coding RNA; ns, not significant.

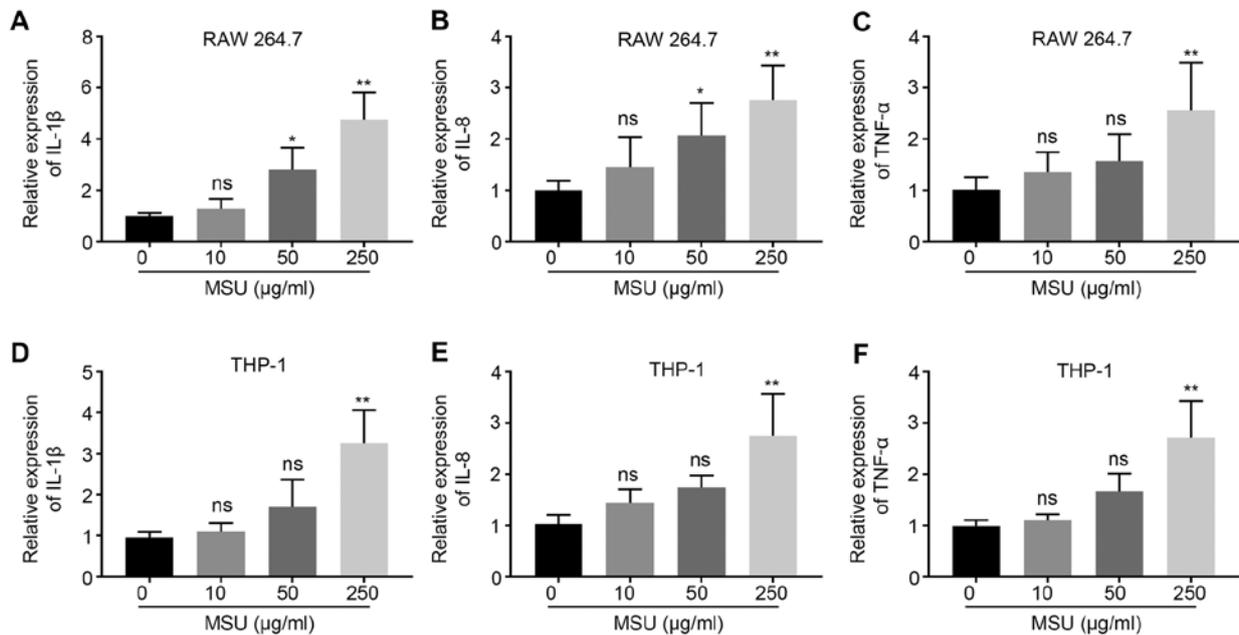


Figure 2. Effect of MSU treatment on the expression of pro-inflammatory cytokines. The mRNA expression levels of inflammatory cytokines, (A) IL-1β, (B) IL-8 and (C) TNFα, were upregulated in RAW 264.7 cells as the concentration of MSU increased. The mRNA expression levels of inflammatory cytokines, (D) IL-1β, (E) IL-8 and (F) TNFα, were upregulated in THP-1 cells as the concentration of MSU increased. *P<0.05, **P<0.01 vs. 0. MSU, monosodium urate; IL, interleukin; TNF, tumor necrosis factor; ns, not significant.

points (0, 6, 12 and 24 h). Compared with baseline (0 h) LPS stimulation, the positive expression rates of lncRNA-MM2P were 72% at 12 h and 35% at 24 h in RAW 264.7 cells, and 42% at 24 h in THP-1-derived macrophages. As the expression of lncRNA-MM2P decreased significantly at 24 h in both RAW 264.7 and THP-1-derived macrophages, this duration was used for subsequent experiments. After treating RAW 264.7 and THP-1-derived macrophages with different concentrations (0, 10, 50, 250 µg/ml) of MSU crystals for 24 h, the positive

expression rates of lncRNA-MM2P were 37% in RAW 264.7 and 32% in THP-1-derived macrophages when 250 µg/ml was used, which was a significant decrease (Fig. 1).

MSU-mediated inflammatory responses in macrophages. RAW 264.7 and THP-1-derived macrophages were treated with different concentrations (0, 10, 50, 250 µg/ml) of MSU crystals for 24 h. Compared with treatment with 0 µg/ml MSU crystals, 50 µg/ml or 250 µg/ml significantly increased inflam-

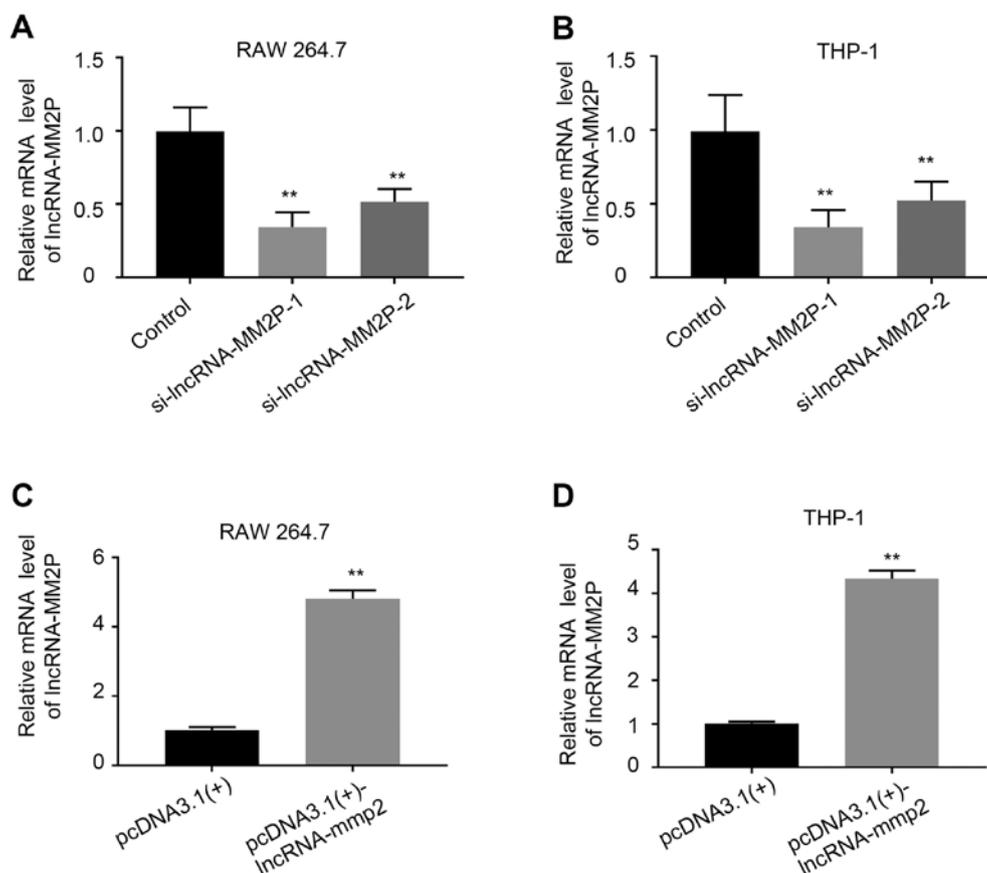


Figure 3. Knockdown and overexpression of lncRNA-MM2P in RAW 264.7 and THP-1 cells. Expression of lncRNA-MM2P is decreased in (A) RAW 264.7 and (B) THP-1 cells transfected with si-lncRNA-MM2P-1 or -2 compared with the control. Expression of lncRNA-MM2P is upregulated in (C) RAW 264.7 and (D) THP-1 cells transfected with pcDNA3.1(+)-lncRNA-MM2P compared with the control. ** $P < 0.01$ vs. control or pcDNA3.1(+). lncRNA, long non-coding RNA; siRNA, small interfering RNA.

matory responses in RAW 264.7 cells, 50 $\mu\text{g/ml}$ MSU crystals led to a 2.8-fold increase in the mRNA expression of IL-1 β and a 1.9-fold increase in the mRNA expression of IL-8; and treatment with 250 $\mu\text{g/ml}$ MSU crystals led to a 4.3-fold, 2.4-fold and 2.1-fold increase in the mRNA expression levels of IL-1 β , IL-8 and TNF α , respectively. In THP-1-derived macrophages, treatment with 250 $\mu\text{g/ml}$ MSU crystals resulted in a 3.2-fold, 2.3-fold and 2.5-fold increase in the mRNA expression levels of IL-1 β , IL-8 and TNF α , respectively (Fig. 2).

Inhibition of lncRNA-MM2P enhanced MSU-mediated inflammatory responses. After transfection with lncRNA-MM2P siRNA-1 or -2, the expression of lncRNA-MM2P significantly decreased in RAW 264.7 and THP-1-derived macrophages, whereas using pcDNA3.1(+)-lncRNA-MM2P significantly increased the expression of lncRNA-MM2P (Fig. 3). As the knockdown efficiency of lncRNA-MM2P siRNA-2 was less than that of lncRNA-MM2P siRNA-1, lncRNA-MM2P siRNA-1 was used in subsequent experiments. Inhibition of lncRNA-MM2P did not influence the inflammatory responses of macrophages without MSU treatment. However, after treatment with 250 $\mu\text{g/ml}$ MSU crystals, RAW 264.7 cells transfected with lncRNA-MM2P siRNA showed a 1.9-fold increase in the mRNA expression of IL-1 β , a 1.4-fold increase of IL-8 (not significant) and a 0.8-fold increase of TNF α mRNA expression level (not significant) compared with RAW 264.7 cells transfected with

scramble siRNA and pre-treated with MSU (control + MSU). Similarly, after treatment with 250 $\mu\text{g/ml}$ MSU crystals, THP-1-derived macrophages transfected with lncRNA-MM2P siRNA showed a 3.6-fold, a 1.9-fold and a 1.4-fold increase in the mRNA expression levels of IL-1 β , IL-8 and TNF α compared with THP-1-derived macrophages transfected with scramble siRNA and pre-treated with MSU (control + MSU). Whereas, transfection using pcDNA3.1(+)-lncRNA-MM2P exerted the opposite effects on these inflammatory factors (Fig. 4).

The supernatants were used to further verify the effects of lncRNA-MM2P in MSU-mediated inflammatory cytokine secretion. Compared with RAW 264.7 cells transfected with scramble siRNA and pre-treated with MSU (control + MSU), after treatment with 250 $\mu\text{g/ml}$ MSU crystals, RAW 264.7 cells transfected with lncRNA-MM2P siRNA showed a 2.1-fold higher IL-1 β concentration, a 2.5-fold higher IL-8 concentration and a 1.2-fold higher TNF α concentration. Compared with THP-1-derived macrophages transfected with scramble siRNA and pre-treated with MSU (control + MSU), after treatment with 250 $\mu\text{g/ml}$ MSU crystals, THP-1 transfected with lncRNA-MM2P siRNA exhibited a 1.8-fold higher IL-1 β concentration, a 1.5-fold higher IL-8 concentration and a 1.3-fold higher TNF α concentration. However, transfection using pcDNA3.1(+)-lncRNA-MM2P significantly decreased the expression of IL-1 β , IL-8 and TNF α in MSU-induced RAW 264.7 and THP-1-derived macrophages (Fig. 5).

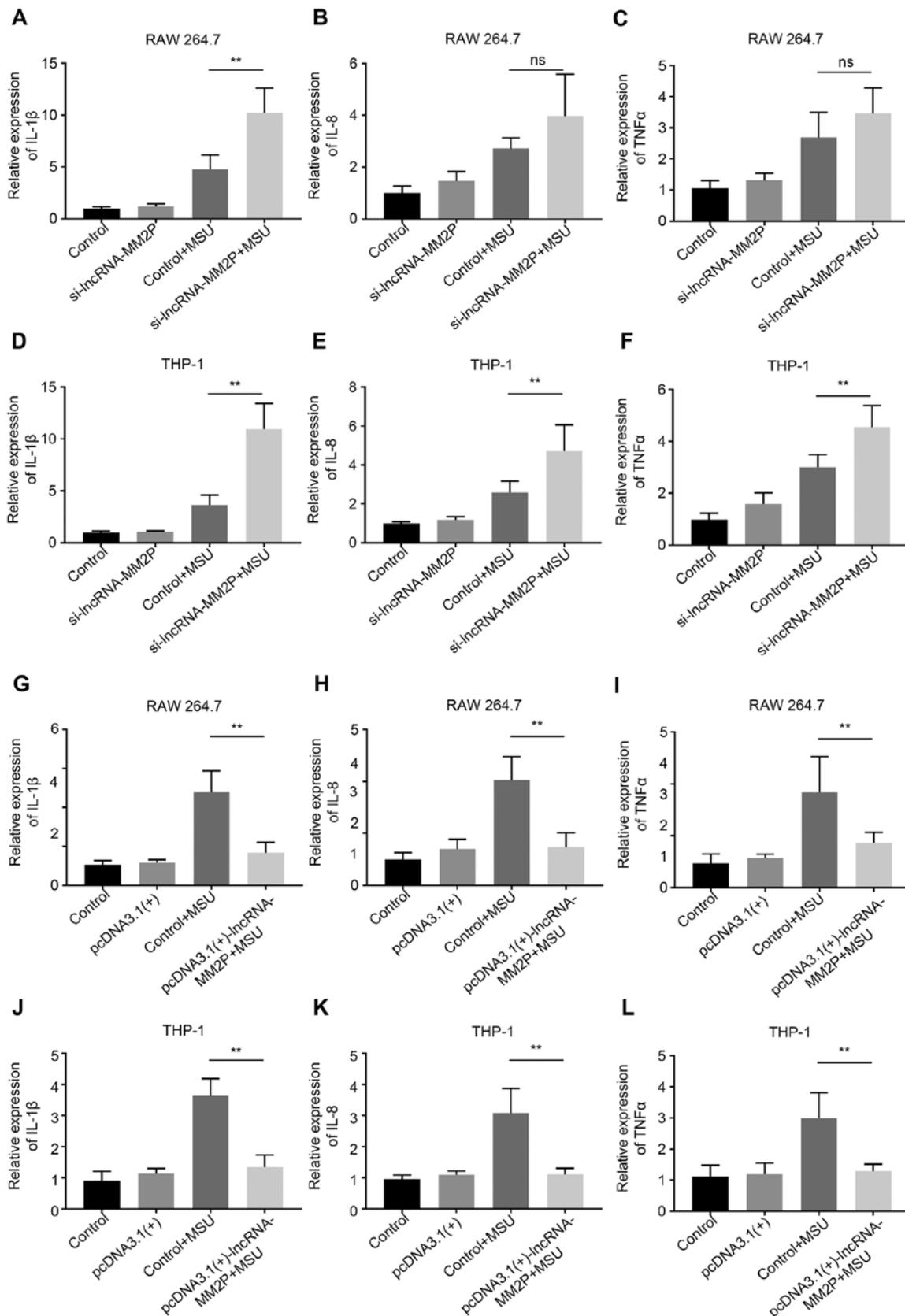


Figure 4. Effect of lncRNA-MM2P on the expression of pro-inflammatory cytokines in MSU-treated cells. The mRNA expression of inflammatory cytokines, (A) IL-1 β , (B) IL-8 and (C) TNF α , increased in RAW 264.7 cells after MSU treatment, and these effects were more notable when lncRNA-MM2P-1 was knocked down in cells. The mRNA expression of inflammatory cytokines, (D) IL-1 β , (E) IL-8 and (F) TNF α , increased in THP-1 cells after MSU treatment, and these effects were more notable when lncRNA-MM2P-1 was knocked down in cells. The mRNA expression of inflammatory cytokines, (G) IL-1 β , (H) IL-8 and (I) TNF α , increased in RAW 264.7 cells after MSU treatment, and these effects were reversed in lncRNA-MM2P-1 overexpressing cells. The mRNA expression of inflammatory cytokines, (J) IL-1 β , (K) IL-8 and (L) TNF α , increased in THP-1 cells after MSU treatment, and these effects were reversed in lncRNA-MM2P-1-overexpressing cells. **P<0.01. lncRNA, long non-coding RNA; MSU, monosodium urate; IL, interleukin; TNF, tumor necrosis factor; ns, not significant.

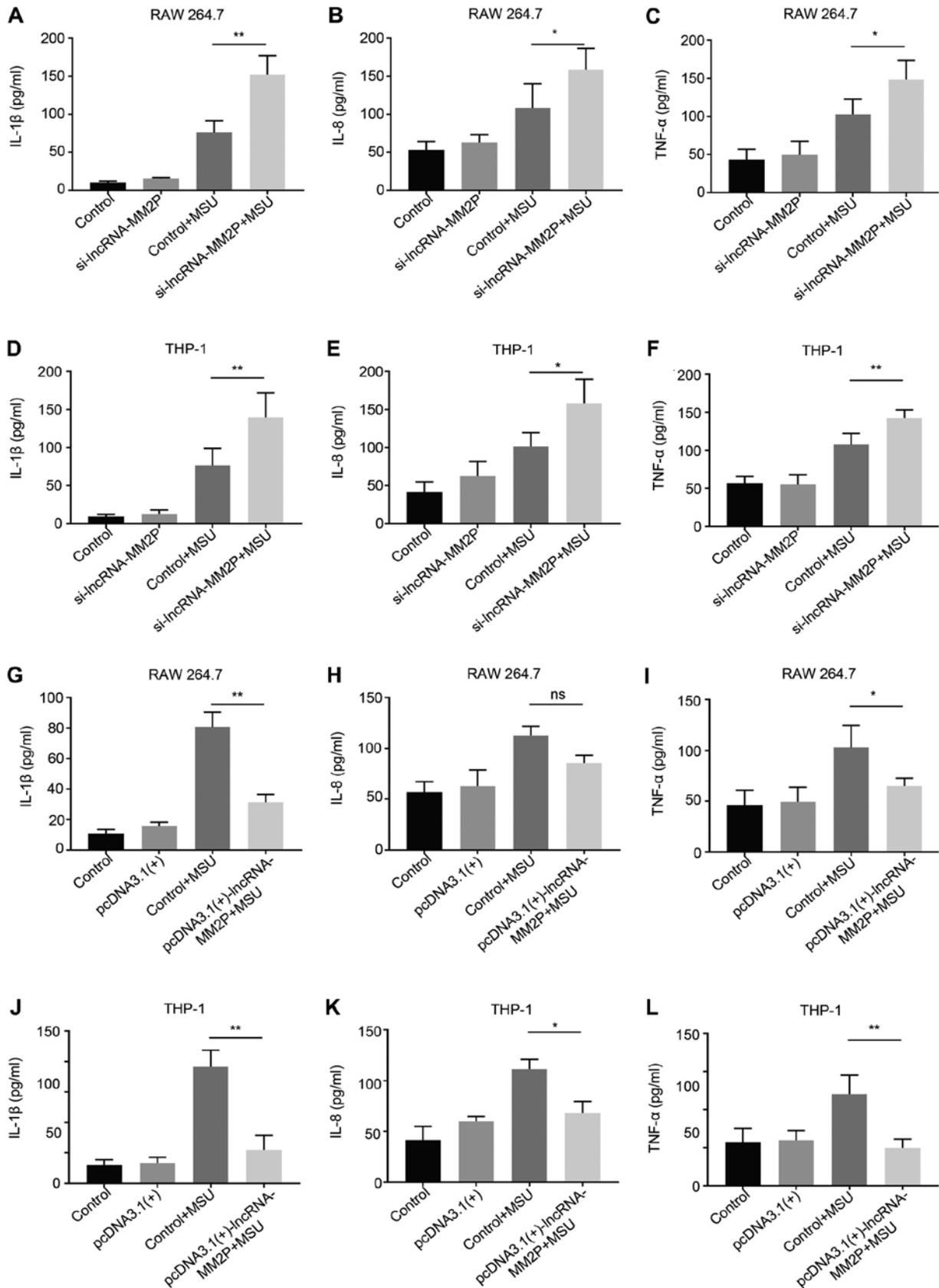


Figure 5. Effects of lncRNA-MM2P on the expression of pro-inflammatory cytokines in the supernatants of MSU-induced cells. The mRNA expression of inflammatory cytokines, (A) IL-1 β , (B) IL-8 and (C) TNF α , increased in the supernatants of RAW 264.7 cells after MSU treatment, and these effects were more notable when lncRNA-MM2P-1 was knocked down in cells. The mRNA expression of inflammatory cytokines, (D) IL-1 β , (E) IL-8 and (F) TNF α , increased in the supernatant of THP-1 cells after MSU treatment, and these effects were more notable when lncRNA-MM2P-1 was knocked down in cells. The mRNA expression of inflammatory cytokines, (G) IL-1 β , (H) IL-8 and (I) TNF α , increased in the supernatant of RAW 264.7 cells after MSU treatment, and these effects were reversed in lncRNA-MM2P-1 overexpressing cells. The mRNA expression of inflammatory cytokines, (J) IL-1 β , (K) IL-8 and (L) TNF α , increased in the supernatant of THP-1 cells after MSU treatment, and these effects were reversed in lncRNA-MM2P-1 overexpressing cells. *P<0.05, **P<0.01. lncRNA, long non-coding RNA; MSU, monosodium urate; IL, interleukin; TNF, tumor necrosis factor; ns, not significant.

Discussion

The pro- and anti-inflammatory functions of macrophages are important for MSU-induced inflammation; however, the mechanism is still unclear. In the present study, it was found that lncRNA-MM2P, a novel modulator of M1/M2 polarization of macrophages, was a key regulator of MSU-induced inflammation, which can trigger the development of AGA. Inhibition of lncRNA-MM2P enhanced MSU-mediated inflammatory responses. Therefore, lncRNA-MM2P may be a potential target in the treatment of AGA.

It is hypothesized that the polarization of macrophages is a dynamic process of development and heterogeneity (19). When macrophages polarize to M1 type, it facilitates the release of inflammatory cytokines, these inflammatory cytokines can also induce the polarization of macrophages to M2 type, thus leading to the inhibition of the inflammatory response (20). It has recently been demonstrated that lncRNAs, which have multiple regulatory functions, are also modulators of polarization. A previous study reported that lncRNA GAS5 has a role in the development of childhood pneumonia by activating M1 macrophage polarization and suppressing M2 macrophage polarization (21). Another study revealed that the knockdown of lncRNA Malat1 leads to M2 activation, which then decreases LPS-induced systemic and pulmonary inflammation and injury (22). Furthermore, it has been demonstrated that lncRNA MIR155HG is highly expressed in granulocyte-macrophage colony-stimulating factor (GM-CSF)-induced macrophages from patients with chronic obstructive pulmonary disease, and overexpression of lncRNA MIR155HG promotes GM-CSF-induced M1 macrophage polarization and inhibits M2 macrophage polarization (23). Among a variety of lncRNAs studied, lncRNA-MM2P was reported to be the only lncRNA upregulated during M2 polarization and downregulated in M1 macrophages at the same time (12). In the present study, it was demonstrated that the expression of lncRNA-MM2P significantly decreased in MSU-treated macrophages, which was accompanied by an increase in inflammatory responses. Knockdown of lncRNA-MM2P in macrophages enhanced MSU-mediated inflammatory responses. The functional characterization of lncRNA-MM2P in MSU-induced inflammation can help generate further insight beyond the traditional physiopathology of AGA.

The mechanism of lncRNA-MM2P-regulated macrophage polarization remains unclear, and STAT6 activation could provide a possible explanation. In a model of macrophage-promoted tumorigenesis, it was revealed that knockdown of lncRNA-MM2P suppressed M2 polarization of macrophages by reducing phosphorylation of STAT6 (12). Furthermore, using a model of MSU-induced inflammation, it was demonstrated that the activation of the PI3K/Akt/STAT6 pathway is associated with decreased M1 macrophage polarization (9). Therefore, we suggest that inhibition of lncRNA-MM2P may lead to a decrease in the expression of phosphorylated STAT6, which aggravates MSU-mediated inflammatory responses and this hypothesis will be our next research objective.

In conclusion, the present results suggest that lncRNA-MM2P, a novel modulator of M1/M2 polarization of macrophages, is an important regulator of MSU-induced inflammation, and could have a role in the development AGA. Further knowledge of the role of lncRNA-MM2P in AGA could contribute to

an improved understanding of the pathogenesis of AGA and may provide further insight into the potential and efficacy of lncRNA-MM2P-based prevention and treatment options.

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Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors' contributions

XZ, YZ and JZ designed all of the experiments and revised the manuscript; YZ and MQ revised the manuscript; SJ, FY, MQ and XW performed the experiments; and JLi, XL and JLe analyzed and interpreted the data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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