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Supplemental Information

MOZ Forms an Autoregulatory Feedback Loop with miR-223 in AML and Monocyte/Macrophage Development

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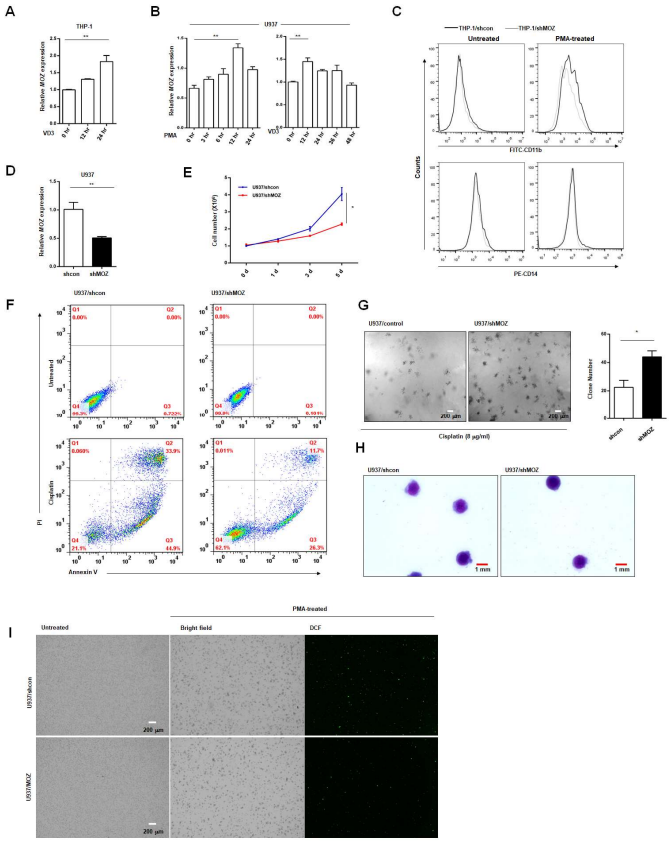

Figure S1 Knockdown of MOZ blocks monocyte differentiation, Related to Figure 2.

(A) THP-1 was treated with 1,25(OH)$_2$-VitD$_3$ (VD3) for indicated periods and then the expression of MOZ was determined by Q-PCR. (B) U937 cells were cultured with PMA or VD3 for indicated times and MOZ expression was examined by Q-PCR. (C) THP-1 was treated with PMA for 24 hours and subsequently be analyzed for detecting the expression of CD11b and CD14 using flow cytometry. (D) The efficiency of MOZ-knockdown in U937 was determined by Q-PCR. (E-I) Knocking down MOZ in U937 resulted in blocking differentiation. Cells numbers were counted at indicated time points for calculating the proliferate rate using trypan blue staining to exclude the dead cells (E). shMOZ and shcon cells were treated with or without cisplatin (30 μg/ml) for 24 hours and then flow cytometry was used for analyzing cells apoptosis which were stained with PI/Annexin V (F). Soft agar assay was used to determine the growth of shcon or shMOZ cells treated with cisplatin for 15 days. The clones were photographed by microscope (G). The morphologic analysis of shcon and shMOZ cells was used with Wright-Giemsa staining (H). The morphology of shcon or shMOZ treated with or without PMA for 24 hours was analyzed by light microscope (left and middle), PMA-stimulated cells were treated with DCFH-DA (10 μM) for 4 hours and subsequently to be analyzed by fluorescence microscope (I, right). Data represent the mean ±SEM from three independent experiments. Similar results were obtained in three independent experiments. **P<0.05; **P<0.01; ***P<0.001, NS, not significant.*

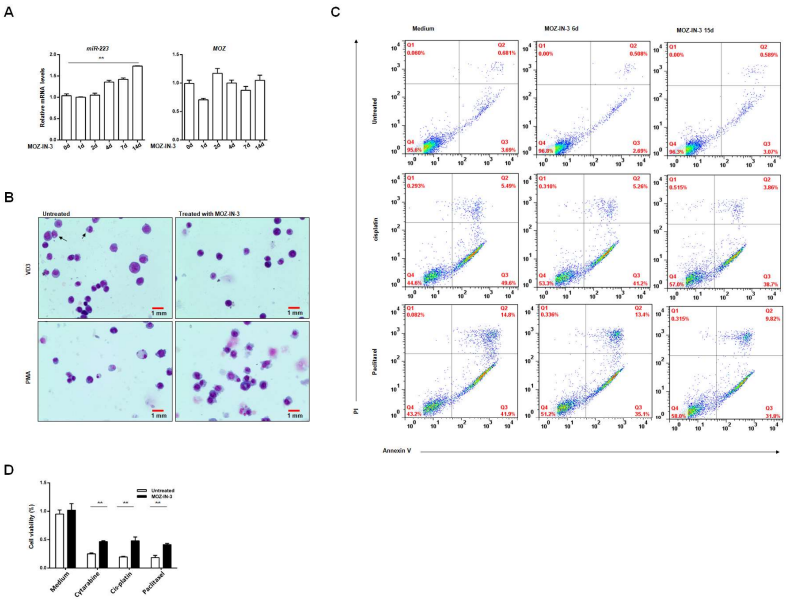

Figure S2 MOZ inhibitor increases the activity of apoptosis resistance to cisplatin induced death, Related to Figure 2.

(A) U937 was treated with MOZ-IN-3 (3μM) for indicated periods and then the miR-223 or MOZ expression were determined by Q-PCR. (B) U937 which was pre-treated with or without MOZ-IN-3 for 15 days was stimulated with PMA (10 μg/ml) or 1,25OH-VitD3 (100 nM) for 24 hours or 48 hours, respectively. Wright-Giemsa staining were used to analyze the morphology of U937. (C-D) U937 treated with or without MOZ-IN-3 was added with cisplatin(C), cytarabine and paclitaxel (D) to analyze the apoptosis rate (C) or cell viability by using flow cytometry or CCK8 assay, respectively. Data represent the mean ±SEM from three independent experiments. Similar results were obtained in three independent experiments. **P<0.05; **P<0.01; ***P<0.001, NS, not significant.*

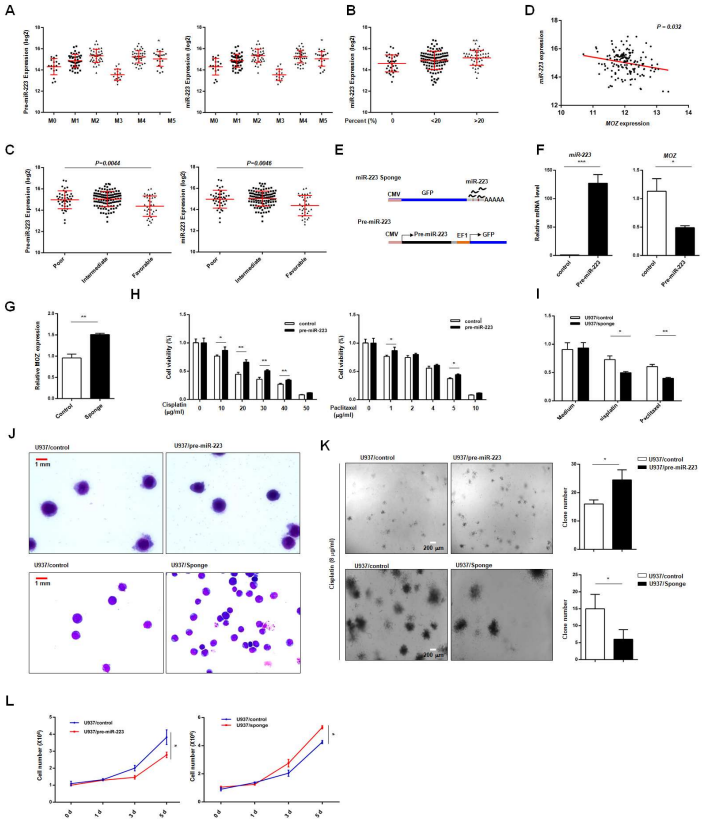

Figure S3 High expression of miR-223 is associated with poor prognosis**,** Related to Figure 1 and Figure 6.

(A) Integrate analysis of miR-223 expression in different subtypes of AML based on TCGA database. (B) Integrate analysis of miR-223 expression in AML cases which were subdivided into 3 groups based on the proportion of monocytes (=0, <20% and >20%). (C) Integrate analysis of miR-223 expression in AML subgroups with poor, intermediate or advanced prognosis. (D) Correlation analysis between MOZ and miR-223 expression. (E) Schematic representation of design for miR-223 overexpression or knockdown. (F-G) Established miR-223 overexpression or knockdown cells were analyzed for the MOZ expression by using Q-PCR. (H-I) miR-223 overexpression or knockdown cells and their corresponding control cells were treated with chemotherapeutic drugs at indicated concentration for 24 hours and then analyzed using CCK8 assay. (J) Wright-Giemsa staining analyzed the morphology of miR-223 overexpression or knockdown cells. (K) miR-223 overexpression or knockdown cells and their corresponding control cells were treated with cisplatin (8 μg/ml) and then seeded into soft agar for 15 days, the visible colonies were photographed by light microscope. (L) miR-223 overexpression or knockdown cells and their corresponding control cells were counted using trypan blue staining and the number of live cells were calculated for growth curve. Data are presented as the mean ±SEM from three independent experiments. Similar results were obtained in three independent experiments. **P<0.05; **P<0.01; ***P<0.001, NS, not significant.*

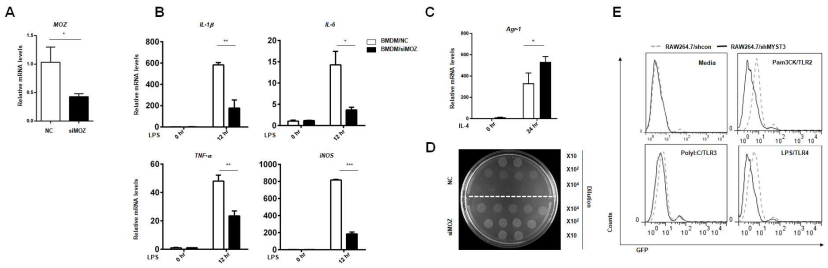

Figure S4 Knockdown of MOZ dampens the M1 activity of BMDM, Related to Figure 4.
(A) BMDMs were transfected with 20 nM siMOZ or control siRNA. At day 3 after transfection, the efficiency of knockdown was determined. (B-C) BMDMs transfected with siMOZ or scramble siRNA were stimulated with LPS (100 ng/ml) or IL-4 (20 ng/ml) for the indicated time periods and subjected to analysis of the levels of M1 markers (B) or the M2 marker Arginase 1 (C) by Q-PCR. (D) siMOZ or control BMDMs were pretreated with LPS for 12 hours. After stimulation, live *E-coli* was added to the media for another 2 hours, and the CFUs of the supernatant were then determined. (E) siMOZ or control BMDMs were treated with TLR agonists at the indicated concentrations together with GFP-bacteria for 4 hours and subjected to FACS analysis for GFP intensity. Data represent the mean ±SEM from three independent experiments. Similar results were obtained in three independent experiments. **P<0.05; **P<0.01; ***P<0.001, NS, not significant.*

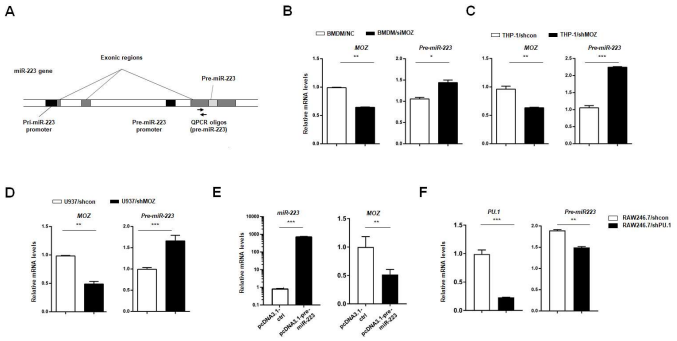

Figure S5 MOZ acts as a target of miR-223 and a repressor for the miR-223 gene, Related to Figure 7.

(A) Schematic representation of the miR-223 gene. (B) RAW264.7 cells were transiently transfected with overexpression plasmids of miR-223 (pcDNA3.1-pre-miR-233) or control plasmids. After 48 hours, MOZ expression was measured by Q-PCR. (C) 20 nM siMOZ or scramble siRNA was transfected into BMDMs. After 48 hours, the precursor of miR-223 was detected by Q-PCR. (D) Stably transfected THP-1 cells with either shPU.1 or control shRNA were examined for miR-223 expression levels by Q-PCR. Data represents the mean ±SEM from three independent experiments. Similar results were obtained in three independent experiments. **P<0.05; **P<0.01; ***P<0.001, NS, not significant.*

Quantitative RT-PCR primers			
Genes	**Forward (5'-3')**	**Reverse (5'-3')**	**Species**
MOZ	TCATCCCATGGCTTGGATCG	GAAGTGCTATTCGCCCAGGA	H
MOZ	CTGTCCAACCAGCCGCCAA	GCTTCCAGACTCGGGTATCTCC	M
PU.1	AATGGAAGGGTTTCCCCTCG	GCTATGGCTCTCCCCATCAC	H
PU.1	GCAGGGGATCTGACCAACCT	AGTCATCCGATGGAGGGGC	M
pre-miR-223	GAAAGCCCAATTCCATCTG	AGTGGAGTGGTGCCTTGGT	H
pre-miR-223	GTCCCCGAGGACAGAATGAT	GGGGATCCTGGGTGTTTGTC	M
RUNX1	GCAGGCAACGATGAAAACTACT	GCAACTTGTGGCGGATTTGTA	M
IL6	CCAAGAGGTGAGTGCTTCCC	CTGTTGTTCAGACTCTCTCCCT	M
IL1B	GCAACTGTTCCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT	M
IL10	GCTCTTACTGACTGGCATGAG	CGCAGCTCTAGGAGCATGTG	M
TNFA	GACGTGGAACTGGCAGAAGAG	TTGGTGGTTTGTGAGTGTGAG	M
iNOS	ATCTTTGCCACCAAGATGGCCTGG	TTCCTGTGCTGTGCTACAGTTCCG	M
Agr1	TGACTGAAGTAGACAAGCTGGGGAT	CGACATCAAAGCTCAGGTGAATCGG	M
GAPDH	CTGGGCTACACTGAGCACC	AAGTGGTCGTTGAGGGCAATG	H
GAPHD	GGTGAAGGTCGGTGTGAACG	CTCGCTCCTGGAAGATGGTG	M
ChIP primers			
TNFA	GAGAAGGCTTGTGAGGTCCG	GGCAGAGCAGCTTGAGAGTT	M
IL1B	GGAGAGCACAGAAGCACCAT	TAGGCACGTAGATGCACACC	M
Repoter primers			
ppri-miR-223^{-760}	AACTCGAGTCCACAAAGGTGCTGTTACAA	AAAAGCTTGAAGTGGTGCCTTTGTCTTG	M
ppri-miR-223$^{PU.1\text{-}del}$	ACTTTATCTTCATGACACCAAGAGTC	GTCATGAAGATAAAGTTGAGGCTAAC	M
ppri-miR-223$^{RUNX1\text{-}mut}$	TAAAACTCAGCTTTCCAGAACCTCTATAGT	TTCTGGAAAGCTGAGTTTTATGTTGTGAGC	M
hMOZ- 3'UTR	CCGCTCGAGCAAGCAGTCACTCAACGG	ATTTGCGGCCGCCCCTCTTCTCATTAGCCAC	H
hMOZ- 3'UTR-mut	CCTTTTATGGGTTTTAACCAGAGAAAAATG	TCTCTGGTTAAAACCCATAAAAGGTTCCTT	H
Clone primers			
pre-miR-223	GAAGATCTCCACCAGAATCTCCAGACAA	GCGTCGACACAGATGCATACCAGACTTG	M

Table S1 The primers used in our present works are shown, Related to Figure 2, Figure 4, Figure 6 and Figure 7.

Transparent Methods

Mice and reagents

C57BL/6 mice were purchased from SIPPR-BK Experimental Animal Ltd. Co. (Shanghai, China). Experiments and animal care were performed according to protocols approved by the University of Science and Technology of China (USTC) Institutional Animal Care and Use Committee. Lipopolysaccharide (LPS), polyI:C and PAM3CK4 were purchased from Sigma (St. Louis, MO). Recombinant human and mouse GM-CSF and M-CSF were from PeproTech (Rocky Hill, NJ). 1,25OH-VitD3 was purchased from Sigma (St. Louis, MO). MOZ-IN-3 was from MCE (MedChem Express (Monmouth Junction, NJ, USA).MiR-223 mimics and control, miR-223 inhibitors and inhibitor control, MOZ small interfering RNA (siRNA) and scrambled control siRNA were purchased from GenePharma (Shanghai, China). CCK8 kit and Wright-Giemsa solution was obtained from Solarbio (Beijing, China). Primary antibodies against p65/phospho-p65 (S560), AKT/phospho-AKT (S473), p38/phospho-p38, Erk/phospho-Erk1/2, and JNK/phospho-JNK were obtained from Cell Signal Technology (Danvers, MA). Antibodies against MOZ were purchased from Abcam. Antibodies against GAPDH and Ac-lysine were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Phosphorylated and acetylated Histone H3 antibody was purchased from Genetex Inc. (California, USA). HRP-conjugated secondary antibodies were obtained from Biolegend. FITC-coupled and PE-coupled CD34 or CD38 antibodies, respectively, for flow cytometry were obtained from BD Biosciences. ELISA kits detecting IL-6 and IL-1β were purchased from Lianke Ct.

Monocyte isolation and cell culture

The study was approved by the institutional ethics board of the University of Science and Technology of China and conducted in accordance with the Declaration of Helsinki. Human monocytes were isolated from the peripheral blood of healthy adult volunteers using consecutive Ficoll-Hypaque and Percoll density gradient centrifugation as reported previously (Todd and Schlossman, 1982). The purity of monocytes was ~94% (± 2%) based on flow cytometry analysis of CD14. Human monocyte-derived macrophages were generated by culturing monocytes in the presence of GM-CSF as described previously (Cassol et al., 2009). Briefly, monocytes were incubated at 5×10^5 cells/ml in RPMI 1640 medium (RPMI 1640 plus 2 mM glutamine, 25 mM HEPES, 100 U/ml penicillin, and 100 mg/ml streptomycin) containing 10% FBS and 100 ng/ml recombinant human or mouse GM-CSF at 37℃ in a 5% CO_2 incubator for 5 days. BMDMs were derived from the bone marrow of C57BL/6 mice. In brief, after lysis of red blood cells, bone marrow cells were cultured in DMEM media containing 10% FBS and 20 ng/ml M-CSF for 5 days to establish macrophages. RAW264.7 and HEK293 cells were maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin. THP-1 cells were cultured in RPMI 1640 supplemented with 10% FBS and standard concentrations of penicillin-streptomycin. To induce M1 polarization, macrophages were cultured in the same cytokine mixture in the presence of LPS (100 ng/ml) for an indicated period of time at 37 ℃ in a 5% CO_2 incubator.

Plasmids and transfection

The library of shRNA used for producing viral-packaged interfering RNA for target genes was obtained from Sigma. The protocol of lentiviral particle production was performed as previously

described (Hornung et al., 2009). For generating stable cell lines, lentiviral particles were added together with Polybrene to 6-well plates that had been seeded with 8×10^5 cells, and the final concentration of Polybrene was 8 μg/ml. Cells were incubated overnight, and the lentivirus/Polybrene mixture was replaced with fresh puromycin-containing culture media every 3-4 days until resistant colonies could be identified. For knocking down MOZ with synthetic siRNA in BMDMs, 6×10^6 cells were seeded into 6-well plates. Lipofectamine 2000 was used to deliver siRNA into cells as per the manufacturer's recommendations.

Luciferase assay

A 1-kb fragment containing the predicted miR-223 binding element in the MOZ 3'UTR was obtained by PCR amplification using human genomic DNA as a template and was then cloned into the Bgl-II and EcoRI sites of the psiCheck2-Reporter Luciferase vector. The resulting construct was designated hMOZ-3'UTR. For mutant MOZ 3'UTR construction, nucleotides in the 3'UTR of the MOZ gene that matched the miR-223 "seed sequence" were mutated by site-directed mutagenesis using site-directed mutagenesis kits (TransGen, Biotech). The resulting construct was designated hMOZ-3'UTR-mut. The sequences of the primers used for PCR amplification are listed in Table S1. hMOZ-3'UTR or -mut was cotransfected with miR-223 overexpression plasmids using Lipofectamine 2000 reagent (Invitrogen). At 24 hours after transfection, the luciferase activity was determined using the Dual Luciferase Assay System (Promega). For analysis of the promoter activity of miR-223, a -761 to +21 bp fragment (relative to the translational start site) containing the confirmed PU.1 binding sites and predicted RUNX1 binding site was amplified from mouse genomic DNA using high-fidelity PCR and cloned into pGL3-basic vectors. pri-miR-$223^{PU.1\text{-}del}$ and pri-miR-$223^{RUNX1\text{-}mut}$ reporter constructs were identical to the wild-type construct except that the PU.1-binding sites were deleted or the predicted RUNX1-binding site was inactivated by substitution of CAGAA at positions +59 to +54, respectively. The related primers are in Table S1. Established reporter constructs were transiently co-transfected with pRL plasmids into RAW264.7 cells, and the lysed supernatant was measured using a dual-reporter Luciferase assay (Promega) to determine the luciferase activity.

Bacterial killing assay

A bacterial killing assay was performed as described previously (Banerjee et al., 2013). Briefly, 0.1×10^6 CFU/ml of *E. coli* (BL21DE3pLysS) was added to media in 48-well plates and incubated at 37℃ for 4 hours. The supernatants were then serially diluted 10 times, and 20 μl of each diluted supernatant was seeded on LB-agar plates. The plates were incubated overnight at 37℃, and bacterial colonies were counted after 16 hours.

Bacterial phagocytosis assay

The bacteria for this assay were previously transformed with pET28-GFP vectors. GFP-expressing bacterial clones were co-cultured with macrophages in 48-well plates. After incubation for 4 hours at 37℃ in a 5% CO_2 incubator, the macrophages were washed twice with cold PBS, and a flow cytometry assay was then performed.

Soft agar and CCK8 assay

Colony formation assay in soft agar were done according with standard protocols. In brief, pre-

warmed 2×RPMI-1640 containing 20% FBS, 200 U/ml penicillin, 200 μg/ml streptomycin and equivalent melted 1.2% SeaPlaque Agarose (Lonza) solution were mixed and transferred into a well (3 ml per well) in a 6-well plates, which was then incubated at room temperature for 30 min to allow the bottom agar layer to solidify. Cell suspension (5×10^3 per well) in RPMI-1640/10% FBS was mixed with equivalent 2×RPIM-1640 containing 20% FBS and same volume of 1.2% agar. The mixture was then plated on the bottom agar layer and the top agar was solidified at room temperature before the plates being put into CO_2-incubator. After about 15 days, the visible colonies were counted by light microscope.

CCK8 assay was performed with standard protocol. Briefly, parental cells (U937) and their transfectants were plated in 96-well plates at a density of 1×10^4 per well in 200 μl of RPMI-1640 with 10% FBS. After 12 hours, different dose of chemotherapeutic drugs were added into 96-well plates as demanded for 24 hours, then 20 μl of CCK8 solution was added per well. After incubation for 2 hours at 37℃, we measured the absorbance of each well at 450 nm by using an ELISA plate reader.

Quantitative real-time PCR

Total cellular RNA was extracted using Trizol reagent (Invitrogen). A quantity of 2 μg of total RNA was used for the reverse-transcription reaction using the First Strand cDNA Synthesis kit (Invitrogen). The obtained cDNA was analyzed using real-time PCR, as previously described (Pulikkan et al., 2010). Primer sequences are listed in Table S1.

Immunoblot analysis

For preparing the whole-cell extract, cell were harvested and lysed in radioimmunoprecipitation assay buffer (50 mM Tris-HCl [pH 7.4], 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mg/ml aprotinin, 1 mg/ml leupeptin, 1 mg/ml pepstatin, 1 mM Na_3VO_4, and 1 mM NaF). The protein concentration of lysate was quantified using a Bicinchoninic Acid Protein Assay Kit (Pierce, Rockford, IL). Cell lysates were loading onto an SDS-PAGE gel and separated by electrophoresis. After transfer of the proteins onto a nitrocellulose membrane, the target molecules were detected by Western blotting using corresponding primary antibodies, HRP-conjugated secondary antibodies, and an ECL Detection kit (Pierce).

Measurement of cytokines and chemokines

The supernatants of cultured macrophages were harvested and kept at -80℃. The concentrations of selected cytokines and chemokines were measured using an ELISA kit (Lianke) or a CBA kit (BD) following standard procedures.

Data mining and bioinformatics analysis

Related gene expression profiles in AML cases were extracted from the TCGA and GEO databases. The normalized expression value of related genes in AML cases was used for correlation analysis and survival analysis (Kaplan-Meier) using SPSS software. The analysis of transcriptional regulation of the *MOZ* gene was based on putative transcription factors predicated by various databases or software (PROMO 3.0, JASPAR, COMPEL, FANTOM and Ensembl) on 3 kb of sequence upstream of the proximal transcription start site of the *MOZ* gene. These predicated transcription factors (the setting threshold was >0.9 or 90 according to the different software used)

were clustered using DAVID 6.8, and co-expression was analyzed using cytoscape software.

Statistical analysis

All experiments were repeated three times. The data are presented as the mean ±SEM. Statistical significance was determined by Student's t test and analysis of variance (ANOVA) using SPSS, with a *P* value less than 0.05 considered statistically significant.