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Supplemental Information

MicroRNA-127 Promotes Anti-microbial Host Defense through Restricting A20-Mediated De-ubiquitination of STAT3

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TRANSPARENT METHODS

Reagents

The antibodies against murine β-actin (3700S), IκBα (4812S), p-IκBα (Ser32) (2859s), p65 (8242S), p-p65 (Ser468) (3039s), JNK (9258s), p-JNK (Thr183/Tyr185) (9255s), ERK (4695), p-ERK (Thr202/Tyr204) (9101s), p38 (9212S), p-p38 (Thr180/Tyr182) (9211s), K63 Ub (5621s), and p-STAT3 (9145s) were from Cell Signaling (Beverly, MA). STAT3 (ab119352), A20 (ab179434), and HA (ab187915) were from Abcam (Cambridge, MA). The inhibitor JSI-124, Thioglycollate and 4',6-diamidine-2-phenylindole dihydrochloride (DAPI) were purchased from Sigma (St. Louis, MO, USA). Dynabeads™ Protein A for Immunoprecipitation (10002D) was purchased from Thermo Fisher Scientific (Waltham, MA). M-CSF and the enzyme-linked immunoassay kits for IL-6, IL-1β and TNF-α were from R&D Systems (Minneapolis, MN). The Dual-luciferase reporter assay kit, the pMIR-REPORT and pRL-TK-Renilla luciferase plasmids were purchased from Promega (Madison, WI).

MicroRNA mimics and inhibitor

The sequences of miR-127 and its complementary strand were obtained from the sanger miRNA database and synthesized by Genepharma (Shanghai, China). Specifically, the sequences of double-stranded miR-127 mimics are 5'-UCGGAUCCGUCUGAGCUUGG

CU-3' and 3'- CCAAGCUCAGACGGAUCCGAUU-5'. The sequence of single-stranded miR-127 anti-miR is 5'-AGCCAAGCUCAGACGGAUCCGA-3'. The chemical modifications include phosphorylation on the 5' end, 2'-O-Me modifications for avoiding RNA-induced silencing complex (RISC) formation and increasing stability, as well as the cholesterol-linkage for enhanced cellular uptake.

Mice

All animal experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and approved by the Animal Care and Use Committee of Nanjing University of Chinese Medicine. Male C57BL/6 mice were obtained from Shanghai Experimental Animal Center of Chinese Academy of

Sciences (Shanghai, China) and further bred in a specific-pathogen-free (SPF) environment.

Preparation of S. aureus

The MRSA strain USA300 was kindly provided by Pro. Feng Xu (School of Medicine, Zhejiang University), and was grown to the stationary growth phase at 37°C in Luria-Bertani broth, then harvested by centrifugation (8000 rpm, 5 min). The bacteria were washed by PBS for 3 times and re-suspended in PBS, of which the densities were estimated at OD600.(Xu et al., 2013)

Staphylococcal infection model (Xu et al., 2013)

For pulmonary bacterial infections, a total volume of 50 μL of PBS containing S. aureus (5×10^6 CFU/mice) was intratracheally administered into mice. In some cases, mice were pretreated with a total volume of 50 μL of PBS containing miR-127 mimic, miR-127 inhibitors, and nonspecific controls (NC) (2mg/kg) respectively. For the mortality studies, the mice were intratracheally instilled with 1×10^8 CFU/mice of S. aureus. The survival rate of mice was monitored every 4 h.

Bronchoalveolar lavage and cell differentiation(Ying et al., 2015)

Briefly, murine trachea was exposed through the midline and cannulated with a sterile needle (1mL). Bronchoalveolar lavage fluid (BALF) was obtained by flushing the lungs three times with 1 mL EDTA/PBS (0·5 mM). After centrifugation, BALF supernatants were stored at -80°C for use. Total cell numbers in BALF were counted by the hemocytometer, and neutrophils in BALF were assessed through immunostaining and flow cytometry.

Cell preparation and culture

The RAW264·7 (ATCC® TIB-71™) and 293T (ATCC® CRL-3216™) cell lines were obtained from American Type Culture Collection and have been authenticated and tested for mycoplasma contamination. RAW264·7 and 293T cells were cultured in DMEM medium containing 10% fetal bovine serum (FBS). For alveolar macrophages (AMs), the collected BALF were centrifuges, washed and plated in 12-well plates, and AMs were

selected by adherence after repeated washing

Plasmids or miRNA transfection

For plasmids transfection, RAW264·7 or 293T cells were transiently transfected with the indicated plasmids using X-treme GENE HP DNA Transfection Reagent (Roche). MiR-127 mimics, miR-127 inhibitors, and non-specific RNA were transfected into macrophages using siPORT^{TM} NeoFXTM transfection reagents (Invitrogen) according to standard protocols. The knock-in or knock-down efficacy was determined by qPCR or immunoblotting assay.

Quantitative PCR

Total RNA was isolated using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer's instructions. First-strand cDNA was synthesized using PrimerScript II 1st Stand cDNA Synthesis Kit (Takara, Tokyo, Japan). Quantitative real-time PCR was performed using SYBR Green PCR Master Mix (TOYOBO) to detect mRNA levels of target genes. The data were normalized to β-actin or U6 and determined by the ΔΔCt method. The primers used in the study were synthesized by Genepharma (Shanghai, China) and their sequences were listed in the supplementary material.

The Primer sequences used for quantitative PCR

Sequences of primers used in the study are listed as follows (forward and reverse): IL-1β: 5'-CTCGTGCTGTCGGACCCAT and 5'-CAGGCTTGTGCTCTGCTTGTGA; IL-6: 5'-CCACTTCACAAGTCGGAGGC and 5'-TGCAAGTGCATCATCGTTGTTC; TNF-α: 5'-ATCCGCGACGTGGAACTGGC and 5'-CCATGCCGTTGGCCAGGAGG; β-actin: 5'-CTCATGAAGATCCTGACCGAG and 5'-AGTCTAGAGCAACATAGCACAG; U6: 5'-CTCGCTTCGGCAGCACA and 5'- AACGCTTCACGAATTTGCGT; miR-127: 5'-GCGGCTCGGATCCGTCTGAGCT and 5'- GTGCAGGGTCCGAGGT; LCN2: 5'-GCAGGTGGTACGTTGTGGG and 5'- CTCTTGTAGCTCATAGATGGTGC; IL-17A: 5'-CCACGTCACCCTGGACTCTC and 5'- CTCCGCATTGACACAGCG; IL-22: 5'-ATGAGTTTTTCCCTTATGGGGAC and 5'- GCTGGAAGTTGGACACCTCAA; IL-23p19: 5'- CAGCAGCTCTCTCGGAAT and 5'- ACAACCATCTTCACACTGGATACG; Reg3β: 5'-

ATGGCTCCTACTGCTATGCC and 5’- GTGTCCTCCAGGCCTCTTT; mS100A8: 5’-TGTCCTCAGTTTGTGCAGAATATAAA and 5’-TCACCATCGCAAGGAACTCC; Reg3γ: 5’-CGTGCCTATGGCTCCTATTGCT and ’-TTCAGCGCCACTGAGCACAGAC; β-Defensin-1: 5’-TAGTCTCTTCATCTGTGTTTTTGCATA and 5’-TTCAGCGCCACTGAGCACAGAC; β-Defensin-3: 5’-CCAGGCTGATCCTATCCAGG and 5’-GTCCCATTCATGCGTTCTCT; β-defensin-4: 5’-TGGCCTCCAAAGGAGATAGACA and 5’-AGGCTGATCCTATCCAAAACACA.

Plasmids construction and dual luciferase reporter assays

The cDNA encoding mouse A20 were inserted into pcDNA3·1 vector (Invitrogen) to construct the expressing plasmid. To construct A20-driven reporter plasmid, a 1500 bp fragment of A20 5’UTR was amplified from murine genomic DNA and cloned into pMIR REPORT Vector (Ambion). MiR-127 mimics, anti-miR-127, or non-specific control (NC) nucleotides were transfected respectively into RAW 264·7 cells using X-treme GENE DNA transfection reagent, along with pMIR-A20 or control pMIR plasmids. Cells were then infected with S. aureus (MOI 1) for 6 h and collected for dual-Luciferase reporter assay (Promega).

Determination of cytokine levels

The levels of TNFα, IL-6, IL-1β, and IL-10 in the cell culture supernatants and BALF were measured by ELISA kits (R&D Systems) according to manufacturers’ instructions.

Immunoblotting and co-immunoprecipitation assay

Cell lysates were prepared by lysis buffer (1% Triton X-100, 1% deoxycholate, 0·1% NaN3) containing protease inhibitor cocktail tablets (Roche Diagnostics). Equal amounts of total protein were separated on 10% SDS-polyacrylamide mini-gels and transferred onto Immobilon PVDF membranes (Millipore). After blocked in Tris-buffered saline with Tween-20 (TBST) containing 5% DifcoTM Skim Milk (BD), membranes were incubated with appropriate primary antibodies overnight, followed by the incubation of secondary antibody conjugated with horseradish peroxidase. The signals were visualized using

Millipore™ Immobilon™ Western Chemiluminescent HRP Substrate (ECL). For immunoprecipitation studies, cell lysates were incubated at 4°C for 2 h with a capture antibody or a control IgG, followed by overnight incubation with Dynabeads™ Protein A. The immunocomplexes were collected by centrifugation, washed with ice-cold PBST (PBS-0.02% Tween-20), and separated by SDS-PAGE. The samples were detected by the standard immune-blotting methods.

Internalization and killing of bacteria

To assess the phagocytic capability, macrophages were incubated with CFSE-labeled S. aureus (MOI 10) at indicated time periods. Infected cells were washed, and extracellular bacteria were eliminated by treatment of lysostaphin (20 μg/mL). Macrophages were then collected and intracellular bacteria loads were quantified by flow cytometry (FACS Calibur, BD Biosciences). To determine the bactericidal capability of macrophages (West et al., 2011), cells were grown on cover slips, and incubated with CFSE-labeled S. aureus (MOI 10) for 2 h. Then, cells were washed and further cultured in fresh medium containing lysostaphin (2 μg/mL) for the indicated time periods. After that cells were fixed and the nuclear were counter-stained with DAPI, followed by the observation using fluorescence microscope. Alternatively, cells subjected to staphylococcal infection were collected, washed and lysed to determine bacterial loads using the plate-dilution methods.

Immunofluorescence staining and confocal microscopy of p65

RAW264.7 cells transfected with MiR-127 mimics, anti-miR-127 or non-specific control (NC) nucleotides for 36 h, and then seeded on slides and stimulated with S. aureus for the indicated time. Cells were then collected, fixed with 100% methanol, washed, and permeabilized in 0.2% saponin. After blocking with 5% bovine serum, cells were stained with primary rabbit anti-p65 overnight at 4°C and then stained with goat anti-rabbit IgG conjugated to Texas Red (Invitrogen), and the nuclei were labeled with DAPI (Invitrogen).

Cells were finally mounted in Vectashield and detected under fluorescence confocal microscopy (LSM confocal microscope; Carl Zeiss, Inc.).

Histologic analysis of lung tissues

For the histological analyses, mouse lung samples were washed thoroughly in PBS, fixed in 4% (wt/vol) formalin and embedded in paraffin; 5 μM sections were then stained with hematoxylin and eosin (H&E) using standard procedures. For the immunostaining, lung sections were deparaffinized, hydrated, and blocked in DPBS with 2% normal goat serum. The slides were then stained with the indicated primary and biotin-conjugated secondary antibodies, followed by incubation with streptavidin-conjugated HRP. Slides were finally incubated with DAB reagent and counterstained with haematoxylin for observation.

Statistical analysis

All of the data, unless otherwise indicated, are presented as the means ± SD of three independent experiments. The statistical significance of the differences between two groups was analyzed with Student's t test. Multiple group comparisons were performed by one-way ANOVA followed by Bonferroni post hoc t test. The Kaplan-Meier survival analysis with a log-rank test was applied to evaluate the survival curve. All of the calculations were performed using the Prism software program for Windows (GraphPad Software). A *p* value of 0·05 or less was considered statistically significant.

REFERENCES

WEST, A. P., BRODSKY, I. E., RAHNER, C., WOO, D. K., ERDJUMENT-BROMAGE, H., TEMPST, P., WALSH, M. C., CHOI, Y., SHADEL, G. S. & GHOSH, S. 2011. TLR signalling augments macrophage bactericidal activity through mitochondrial ROS. *Nature,* 472**,** 476-80.

XU, F., KANG, Y., ZHANG, H., PIAO, Z., YIN, H., DIAO, R., XIA, J. & SHI, L. 2013. Akt1-mediated regulation of macrophage polarization in a murine model of Staphylococcus aureus pulmonary infection. *J Infect Dis,* 208**,** 528-38.

YING, H., KANG, Y., ZHANG, H., ZHAO, D., XIA, J., LU, Z., WANG, H., XU, F. & SHI, L. 2015. MiR-127 modulates macrophage polarization and promotes lung inflammation and injury by activating the JNK pathway. *J Immunol,* 194**,** 1239-51.

Supplementary Figures

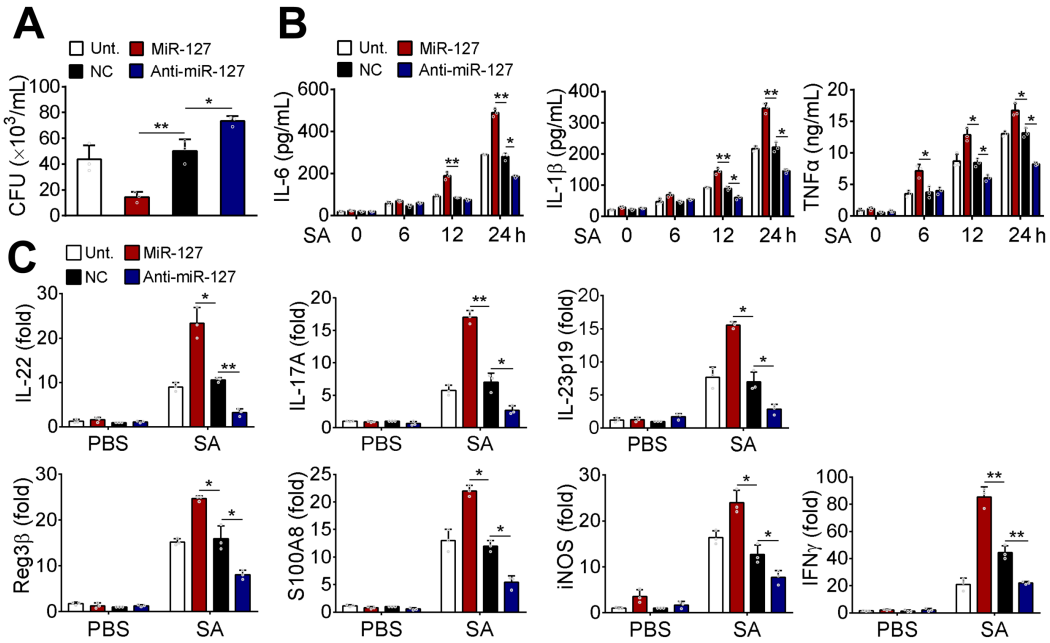

Figure S1. MiR-127 augments anti-microbial response in murine BMDMs. Related to Figure 1. BMDMs were transfected with either miR-127, anti-miR-127 or their non-specific (NC) controls respectively for 24 h, followed by staphylococcus infection (MOI=1) for the indicated time periods. **(A)** Bacterial loads. **(B)** The level of proinflammatory cytokines. **(C)** qPCR analysis of AMPs levels. Results are from at least three independent experiments and expressed as mean ± SD. *p < 0·05, **p < 0·01 by Student's t test.

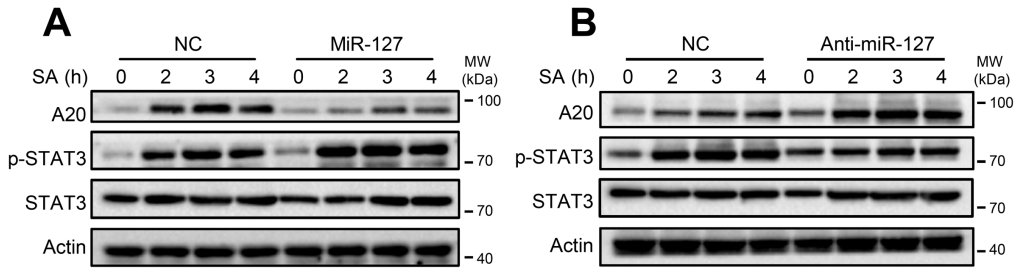

Figure S2. MiR-127 enhances the level of A20 and p-STAT3 (Tyr 705) in BMDMs in response to *S. aureus* infection. Related to Figure 4. BMDMs were transfected with either miR-127, anti-miR-127 or their non-specific (NC) controls respectively for 24 h, followed by staphylococcus infection (MOI=1) for the indicated time periods. **(A, B)** Protein levels of A20, total and phosphorylated STAT3 detected in BMDMs that were transfected with either miR-127 mimic (A) or anti-miR-127 (B) and non-specific control followed by S. aureus (SA) infection.