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MiR-127 Modulates Macrophage Polarization and Promotes Lung Inflammation and Injury by Activating the JNK Pathway

Hangjie Ying,* Yanhua Kang,* Hang Zhang,* Dongjiu Zhao,* Jingyan Xia,† Zhe Lu,* Huanhuan Wang,* Feng Xu,‡ and Liyun Shi*

A polarized macrophage response is presumed to have a pivotal role in a variety of immunological pathophysiology. However, the molecular mechanism underlying macrophage functional shaping remains largely unknown. In this study, we reveal a pivotal role of miR-127 in macrophage development and thereby the pathogenesis of inflammation and lung injury. In particular, miR-127 was demonstrated to be prominently induced upon TLR engagement and repressed by the M2-prone cytokines. Enforced expression of miR-127 in macrophages resulted in significantly increased production of proinflammatory cytokines, whereas deletion of miR-127 impaired M1 gene expression and led to a M2-biased response. Accordingly, intratracheal administration of miR-127 resulted in an exaggerated pulmonary inflammation and injury. Conversely, antagonizing of miR-127 suppressed production of the proinflammatory cytokines and rendered the mice more refractory to the inflammation-associated pathology. Mechanistically, miR-127 demonstrated to target B cell lymphoma 6 (Bcl6) and remarkably downregulated its expression and subsequently dual specificity phosphatase 1 (Dusp1), which in turn enhanced the activation of JNK kinase and hence the development of proinflammatory macrophages. Thereby, reconstitution with the expression of Bcl6 or Dusp1 or inhibition of JNK activity impaired miR-127-mediated skewing of M1 proinflammatory macrophages, whereas interference of Bcl6 or Dusp1 expression abrogated the anti-inflammatory property of anti–miR-127. Together, these data establish miR-127 as a molecular switch during macrophage development and as a potential target for treatment of inflammatory diseases. The Journal of Immunology, 2015, 194: 000-000.

Macrophages are presumed to be a major player in the initiation, propagation, and resolution of inflammation. Triggering the activation of macrophages is critical to eliminate the invading pathogens or the insulting stimulation. However, once the infectious or injurious agents are cleared, the inflammatory signaling must be resolved to prevent the self-destruction. Thus, equal importance should be attached to both the proper activation of macrophages and the transition from an inflammatory to immunosuppressive state (1, 2).

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Abbreviations used in this article: AM, alveolar macrophage; BALF, bronchoalveolar lavage fluid; Bcl, B cell lymphoma; Dusp, dual specificity phosphatase; IC, immune complex; miRNA, microRNA; MPO, myeloperoxidase; NC, nonspecific control; siRNA, small interfering RNA; SOCS1, suppressor of cytokine signaling 1; UTR, untranslated region; ZF, zinc finger.

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system, miRNA has been recognized as an integral part of the fundamental biological processes ranging from development, oncogenesis to cell proliferation, and differentiation (10, 11). Recent studies have begun to unravel the role of miRNA in regulation of the activation and differentiation of macrophages. MiR-125 was reported to promote M1 macrophage polarization and significantly increase the responsiveness of macrophages to IFN-γ. This effect was most likely achieved though miR-125–mediated downregulation of IFN regulatory factor 4, a master regulator for M2 phenotype development (12). Likewise, miR-155 was induced by IFN-γ and TLR agonist and promoted the activation of proinflammatory macrophages through repression of the negative regulators, including suppressor of cytokine signaling 1 (SOCS1) and SHIP1 (13). Additionally, miR-155 exhibited the ability to inhibit the M2-specifying factor, C/EBPβ and IL-13R, and thereby suppressed the alternatively activated macrophage development, suggesting a mutually exclusive modulation of M1/M2 polarization by miR-155 (14). Contrasting with these proinflammatory miRNAs, miR-124 repressed the production of proinflammatory cytokines while elevating the expression of M2-associated genes TGF-β1, Arg1, and FIZZ1. By inhibiting the transcription factors C/EBP-α and PU.1, miR-124 shifted macrophages from an activated phenotype into a quiescent state, conveying the protection for the mice against experimental autoimmune encephalomyelitis (15, 16). Thus, the importance of miRNAs in determining macrophage phenotype and inflammatory disorders has been increasingly appreciated. However, our current knowledge about miR-mediated modulation of macrophage development still remains insufficient, and the fundamental question of how this modulation is integrated into macrophage-associated immunopathology has yet to be investigated (10, 11, 17).

MiR-127 was previously found to be highly expressed in embryos and implicated in the lung development, placental formation, and cellular apoptosis. Aberrant expression of miR-127 was proved to link to cancer of prostate, bladder, and colon (18, 19). Of note, recent studies indicated that miR-127 was prominently induced in the inflammation-related pulmonary disorders such as lung fibrosis, bleomycin, or immunocomplex-induced lung injury and inflammation (20, 21), suggesting a potential role of miR-127 in the inflammatory signaling and lung pathology. However, the exact role of miR-127 and the mechanism involved have been largely undefined. Acute lung injury is one of the prevailing inflammatory lung disorders characterized by exorbitant production of proinflammatory mediators, accumulation of inflammatory cells, and deposition of fibrin and edema fluid (22). Sustained activation of proinflammatory macrophage is thought to contribute substantially to the pathogenesis of acute lung injury. However, the failure to convert proinflammatory M1 into anti-inflammatory M2 phenotype also accounts for the progression of the inflammatory pathology (23).

In the current study, we established a direct linkage between miR-127 and macrophage functional shaping and identified a miRNA-mediated modulation of pulmonary inflammatory disorders. We demonstrated that miR-127 promoted M1-skewing macrophage development and simultaneously repressed the transcription of M2 marker genes. Mechanistic study indicated that miR-127 downregulated the expression of B cell lymphoma (Bcl)6 and hence dual specificity phosphatase (Dusp)1, which in turn enhanced JNK activation and proinflammatory M1 macrophage development. To be functionally relevant, the mice receiving miR-127 mimic displayed the exaggerated lung inflammation and injury, as well as the increased susceptibility to endotox shock. Conversely, antagonizing of miR-127 or silencing of the related signaling molecules proved to reprogram macrophages to the M2 phenotype and thus conveyed the protection of mice against pulmonary and systemic inflammatory disorders. We therefore establish a previously unknown role for miR-127 as a molecular switch in macrophage development and thereby a critical modulator in inflammatory disorders.

Materials and Methods

Reagents

Unless otherwise indicated, all of the Abs were obtained from Cell Signaling. Anti-Bcl6 and anti-actin were both purchased from Santa Cruz. The inhibitors, including U0126 (ERK1/2), SB203580 (p38), PDTC (NF-κB), SP600126 (JNK), and wortmannin (PI3K), were all purchased from Calbiochem. The fluorescein-conjugated mAbs (F4/80, MHC II, CD206, CD11c, and CD11b) and the isotype controls were from BD Pharmingen (San Diego, CA). LPS (055:B5) and poly(IC) were purchased from Sigma-Aldrich, Cpg (5′-ggGgTCAAGgTTGAgggggg-3′) and Pam3CSK4, were both obtained from Invitrogen. The miR-127 mimic, anti–miR-127, and the controls were obtained from GIMA (Shanghai, China) (24).

Animal experiments

All of the animal experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and with the approval of Animal Care and Use Committee of Shanghai Medical College at Hangzhou Normal University. The mice were anesthetized i.p. with ketamine hydrochloride (100 mg/kg) and xylazine (10 mg/kg). A total volume of 50 μl nonspecific oligonucleotides (nonspecific controls [NC]), miR-127 mimic, or anti–miR-127 (2 mg/kg) was instilled intratracheally (25). Twenty-four hours later, PBS or LPS (1 mg/kg, intratracheally) was administered for the indicated time periods. The mice were sacrificed 12 or 24 h post-LPS exposure and subjected to the subsequent functional analysis. For the mortality studies, the mice were pretreated with miR-127 mimic, anti–miR-127, or the controls were injected with a low dose (6 mg/kg, i.p.) or high dose (12 mg/kg) of LPS and monitored twice daily for up to 6 d (26). In short-term studies, blood was collected from mice 3 h after LPS injection, and serum levels of cytokines were measured.

Cell culture and generation of peritoneal macrophages and alveolar macrophages

The RAW264.7 cell lines were obtained from the American Type Culture Collection and grown in DMEM (Life Technologies) containing 10% (v/v) heat-inactivated FBS. To prepare murine peritoneal macrophages, 8-wk-old mice were injected i.p. with 3% thioglycolate broth. After 72 h, the peritoneal cells were harvested, and macrophages were enriched by quick adherence. For alveolar macrophage (AMs), the collected bronchoalveolar lavage fluid (BALF) was plated in 12-well plates, and AMs were selected by adherence after repeated washings (27).

RNA interference

Small interfering RNA (siRNA) targeting mouse Dusp1 or Bcl6 were synthesized by GIMA. siRNA duplexes were transfected into macrophages using siPORT NeoFX transfection reagents (Invitrogen) according to the manufacturer’s protocol, and SYBR Green PCR Master Mix (Bio-Rad) was used to detect the miRNA levels. The expression of selected miRNA was quantified by two-step quantitative real-time PCR (Applied Biosystems). The relative expression levels were determined by applying the ΔΔ cycle threshold method using β-actin or U6 as endogenous controls.

Bronchoalveolar lavage, cell differentiation, and histological analysis (24)

Briefly, the trachea was exposed through a midline incision and cannulated with a sterile 22-gauge needle. BALF was obtained by flushing three times with 1 ml 0.5 mM EDTA/PBS. After centrifugation, the supernatants were stored at −80°C until use. The total cell numbers in the BALF were counted with a hemocytometer, and the differential cell counts were determined on cytospin preparations with Diff-Quick staining (Thermo Fisher Scientific). Alternatively, the neutrophils and macrophages in the
BALF were assessed through immunostaining and flow cytometry. For the histological analyses, mouse lung samples were washed thoroughly in PBS, fixed in 4% (v/v) formalin, and embedded in paraffin; 5 μM sections were then stained with H&E using standard procedures.

**Primers for the quantitative PCR**

The following primers were used: TNF-α, forward, 5′-AAGGCGGG- GTGTCCCTGAG-3′ and reverse, 5′-AGCCAGGTGGGGCAAGC-3′; IL-6, forward, 5′-CACCTCACAAGCTGGAGCTTA-3′ and reverse, 5′-AGTGCATCATCGTGTTCCTAC-3′; IL-1β, forward, 5′-AACCTCACT- TACCAGCAGCGACTTCA-3′ and reverse, 5′-TGTAAGGAAAGACCGCA- CACC-3′; inducible NO synthase, forward, 5′-CCCTGGAGATTTTCTAGGAGCAGG-3′ and reverse, 5′-CGGTCGTACAGGCTGTCCTTGG-3′; Bcl6, forward, 5′-GCGTCTGAGGATGACCGCATT-3′ and reverse, 5′-CGGAAGATGAGAGATTGCCCTGC-3′; and Dusp1, forward, 5′-GCCGAAAAAGCTTTCAATCCT-3′ and reverse, 5′-CGCGTCTAC- GAAGCTTCC-3′.

**Immunoblotting analysis**

Cell lysates were prepared by lysing 2 × 10⁶ cells in lysis buffer (1% Triton X-100, 1% deoxycholate, 0.1% Na₂O₃) containing protease inhibitor mixture tablets (Roche Diagnostics). Equal amounts of protein were separated on 10% SDS-polyacrylamide mini-gels and transferred to Immobilon polyvinylidene difluoride membranes (Millipore). After blocking in TBST containing 5% BSA, the membranes were incubated with the appropriate primary Ab, followed by a secondary Ab conjugated to HRP (Santa Cruz Biotechnology). The signals were visualized with an ECL Western blotting kit (Amersham Biosciences).

**Plasmid transfection and luciferase reporter assays**

The cDNA encoding mouse Bcl6 or Dusp1 was inserted into the pCDNA3.1 vector (Invitrogen). The two cysteine residues of the zinc finger (ZF3) and ZF5 motifs were targeted by PCR mutagenesis using the QuickChange Site-Directed Mutagenesis Kit (Stratagene) (28). The intact or mutant Bcl6 constructs were transfected into RAW cells along with a luciferase construct containing the Dusp1 promoter by JetPEI transfection reagents (PolyPlus). To detect the effect of miR-127 on Bcl6 expression, Bcl6 3′ untranslated region (UTR) was amplified from genomic DNA and cloned in pMIR-REPORT vector (Ambion). pMIR-Bcl6-mut was generated by PCR-based site-directed mutagenesis to delete miR-127 recognition sequence. pMIR-Bcl6, pMIR-Bcl6-mut, or the control vectors were cotransfected with the miRNAs into RAW cells using siPORT NeoFX liposomes (Ambion). Twenty-four hours later, the cells were harvested and assayed for firefly and Renilla luciferase activities.

**Chromatin immunoprecipitation with quantitative PCR**

The cells were cross-linked with 1% formaldehyde, lysed, and sheared with a Diagenode Bioruptor to obtain the chromatin fragments with the size of 200–1000 bp. Chromatin was immunoprecipitated with the Ab for Bcl6 or the control IgG (Santa Cruz Biotechnology) (29). After complete washing, the immunoprecipitated DNA was quantified by real-time PCR. The sequences of the promoter-specific primers are the following: forward, 5′-TTTGGGGGGCGCCAAAGTCTC-3′ and reverse, 5′-ACGGAGGCGGGTTTTAATG-3′. The enrichment was calculated relative to the input.

**Flow cytometry**

Cells were washed in ice-cold flow cytometry buffer (2% [v/v] FCS and 2 mM EDTA in PBS, pH 7.5). After blocking of the unspecific binding, cells were incubated with the fluorescence-conjugated Ab for 30 min and washed three times with flow cytometry buffer. Appropriate isotype controls were used where applicable. Flow cytometry events were gated based on forward and side scatter, and F480 cells were then selected for the analysis of the M1/M2 marker (30). Data were acquired on a FACSCalibur flow cytometer (BD Biosciences) and analyzed with FlowJo (Tree Star).

**Determination of cytokine and myeloperoxidase levels**

The levels of TNF-α, IL-6, and IL-10 in the culture supernatants, serum, or BALF were measured by ELISA (R&D Systems). The lung myeloperoxidase (MPO) levels were determined using mouse MPO ELISA (Hyuck Biotech) following the manufacturer’s instructions.

**Statistical analysis**

All of the data, unless otherwise indicated, are presented as the means ± SD of independent experiments. The statistical significance of the differences between two groups was analyzed with Student t test. A p value ≤0.05 was considered to be statistically significant. All of the calculations were performed using the Prism software program for Windows (GraphPad Software).

**Results**

**Kinetics of miR-127 expression in response to TLR ligands**

To understand its possible function in the inflammatory setting, we first analyzed the expression of miR-127 by macrophages in response to various TLR ligands, including LPS, Pam3CSK₄, poly(I:C), and CpG. The result showed that engagement of TLRs on murine macrophages, either RAW264.7 or primary macrophages, resulted in a unaminous expression profile of miR-127 that was characterized by an initial and transient repression during the first 2-h period, followed by a rapid and sustained induction peaking at 16 h post-exposure. A slight decline in the miR-127 level was observed at the later phase of incubation (Fig. 1A, Supplemental Fig. 1A). This featured expression of miR-127 was also observed in macrophages infected with *Staphylococcus aureus* or vesicular stomatitis virus (Fig. 1B, Supplemental Fig. 1B). Interestingly, we found that a lower dose of TLR ligands generated more significant induction of miR-127 than a higher dose (Fig. 1C), suggesting that there might be a more pressing requirement of miR-127 for the weaker stimuli to trigger macrophage activation. Of note, the expression of miR-127 was revealed to be suppressed by IL-4 or TGF-β/IL-10, the prototype stimuli for M2 macrophages, indicating the differential effects on miR-127 production by M1 or M2 stimuli (Fig. 1D). Furthermore, the in vivo induction of miR-127 was revealed in the lungs, and particularly in AMs, from the mice with intra-thecal instillation of LPS (Fig. 1E, 1F).

Next, to understand the molecular basis for the induction of miR-127 in macrophages, we assessed the possible involvement of some of the key signaling components downstream of TLR. The results showed that LPS-induced miR-127 expression was remarkably suppressed by deletion or inhibition of NF-κB (Fig. 1G), whereas blocking of PI3K or deletion of Akt moderately enhanced its generation (Supplemental Fig. 1C). Also, the level of miR-127 was augmented by inhibition of p38 or JNK kinase, but not affected by ERK inhibitor, suggesting the differential requirements of MAPK kinases for the miR-127 induction in response to LPS (Fig. 1H). Together, these results demonstrated that miR-127 was induced by multiple stimulations of TLR ligands, which was largely dependent on NF-κB signaling.

**MiR-127 promotes M1 signature gene expression while repressing M2 marker gene transcription**

We then sought to determine the functional significance of miR-127 in macrophage response. To this end, RAW264.7 cells were transfected with miR-127 mimic, anti-miR-127, or their NC, respectively, by miR-127 or anti-miR-127 (Fig. 2E). Moreover, the expression of anti-inflammatory cytokine IL-10 was repressed or increased, respectively, by miR-127 or anti-miR-127 (Fig. 2F). The data thus indicated that miR-127 might play a role in determining M1/M2 macrophage differenti-
MiR-127 renders mice more susceptible to endotoxin-induced lung injury and sepsis

Next, we assessed the in vivo function of miR-127 using a murine model of lung inflammation and injury induced by LPS (26). The data showed that, compared with the control animals, the mice pretreated with miR-127 exhibited more severe alveolar damage, as evidenced by the increased interstitial edema and debris deposit in the air space (Fig. 3A). Also, cell infiltration and protein concentrations in the BALF, as well as MPO activity in lung tissues, were remarkably elevated in the mice receiving miR-127 mimic (Fig. 3B–D). The production of IL-6 and TNF-α in BALF was increased, whereas IL-10 level was reduced upon miR-127 treatment (Fig. 3E). Because AMs serve as a major player in the pathogenesis of lung inflammation and injury, we thus evaluated the role for miR-127 in a murine model of endotoxic shock. As shown in Fig. 3I, among the mice challenged with a low dose of endotoxin, up to 60% lethality was induced in the mice pretreated with miR-127 as compared with the 100% survival rate in NC-treated mice. In sharp contrast, inhibition of miR-127 resulted in a profound decrease in the mortality of mice that were challenged with a high dose of endotoxin. Congruent with this observation, the serum levels of IL-6, TNF-α, and IL-1β were significantly augmented in miR-127–treated mice but reduced in miR-127–antagonized animals. Higher level of IL-10, however, was generated in the mice receiving anti–miR-127 (Supplemental Fig. 3A, 3B). Taken together, miR-127 overexpression rendered mice more susceptible to acute lung inflammation and injury, as well as a systemic inflammatory response, which appeared to be closely related with its induction of the proinflammatory M1 macrophages.

JNK is selectively induced by miR-127 to skew the macrophage response

We then sought to determine the mechanism used by miR-127 to regulate macrophage polarization and the related immunopathology. The initial study showed that enforced expression of miR-127 in LPS-stimulated macrophages caused a moderate increase in the phosphorylation of p65 and the upstream kinase, IκB kinase, indicating a promoting effect of miR-127 on NF-κB signaling (Fig. 4A, 4B). However, it has still remained controversial regarding the role of NF-κB in macrophage differentiation, as someone supported the M1-prone effect of NF-κB, whereas others argued that NF-κB was equally important in priming M1 or M2...
gene transcription or even had a negative effect on M1 macrophage development (32–34). We thus turned to other major signaling pathways such as MAPKs, which prove to play an essential role in mediating inflammatory response upon TLR engagement.

Intriguingly, it revealed that, among the three major MAPKs downstream of TLR4, only JNK activity was significantly strengthened by miR-127 in LPS-stimulated macrophages. And inhibition of miR-127 consistently compromised the activation of JNK (Fig. 4C–F).

**FIGURE 2.** miR-127 promotes the expression of proinflammatory cytokines and represses M2-featured gene transcription. RAW cells were treated with miR-127, anti–miR-127, or their negative control (NC) for 24 h, and then subjected to LPS stimulation for the time periods, as indicated. (A and B) Relative mRNA and (C and D) protein levels of proinflammatory cytokines were assayed by quantitative PCR or ELISA, respectively. (E–G) Relative mRNA levels of IL-10 or M2-related genes were examined by quantitative PCR. (H) Expression of MHCII and CD206 on RAW cells 12 h post-LPS exposure was detected by flow cytometry. Representative histograms and average relative mean fluorescence intensity (MFI) are depicted. All the results are from at least three independent experiments and presented as mean ± SD. *p < 0.05, **p < 0.01 by Student t test.
To further determine the functional relevance of JNK activity in this context, we examined its role in the regulation of M1/M2 gene transcription upon miR-127 treatment. Strikingly, blocking of JNK kinase was demonstrated, to a large extent, to abolish miR-127–mediated increase in IL-6 and TNF-α production and relieve its repression on the transcription of IL-10 and M2-featured genes (Fig. 4G–L). Thereby, we revealed a selectively enhanced activation of JNK pathway by miR-127, which was most likely responsible for the skewing of proinflammatory macrophage development.

Dusp1 is responsible for the miR-127–mediated regulation of JNK

Next, we explored the mechanism underlying miR-127–mediated modulation of JNK activity during macrophage activation and differentiation. Because the phosphatase Dusp1 has been proposed to negatively regulate JNK activation and have a protective effect on acute lung injury (35, 36), we then investigated the possible involvement of Dusp1 in miR-127 regulatory loop. Indeed, it indicated that miR-127 reduced, whereas anti–miR-127 heightened, the expression of Dusp1 in LPS-stimulated macrophages (Fig. 5A, 5B), suggesting a negative regulation on Dusp1 by miR-127. Moreover, intratracheal administration of miR-127 or anti–miR-127 resulted in a marked decrease or increase, respectively, in the Dusp1 levels in murine lungs following LPS challenge (Fig. 5C, 5D). Importantly, it was found that interference of Dusp1 with specific siRNA led to enhanced phosphorylation of JNK, whereas enforced expression of Dusp1 abolished miR-127–mediated up-regulation of JNK activation (Fig. 5E). The results thus revealed

**FIGURE 3.** Mice pretreated with miR-127 are highly susceptible to LPS-induced inflammation and acute lung injury. C57BL/6 mice (n = 5 mice/group) were intratracheally injected with miR-127 or NC for 24 h, and challenged with PBS or LPS (1 mg/kg, intratracheally). Twelve hours later, mice were sacrificed and subjected to the functional analysis. (A) Representative H&E staining of lung sections; original magnification ×200. (B) BALF cell counts; (C) MPO level in lungs; (D) total protein level; and (E) cytokine concentration in the BALF. (F and G) mRNA levels of M1- or M2-related genes in the isolated AMs. (H) Representative flow cytometry histogram and average relative mean fluorescence intensity (MFI) for MHCII (left) and CD206 (right) on AMs. (I) Survival rate of the mice that were pretreated with miR-127 or NC and then challenged with LPS at a low dose (6 mg/kg, i.p.), or pretreated with anti–miR-127 or NC and then challenged with LPS at a high dose (12 mg/kg, i.p.). Kaplan–Meier survival plots were depicted (n = 10–12 mice/group). All the data are expressed as the means ± SD (n = 5). *p < 0.05, **p < 0.01 by Student t test (B–H).
a previously unknown regulatory circuit through which miR-127 negatively regulated the phosphatase Dusp1, which in turn caused the enhanced JNK activation and thereby the skewing of the proinflammatory macrophages.

**MiR-127 targets Bcl6 to regulate the expression of Dusp1**

Despite the observation that miR-127 modulated Dusp1 expression in macrophages, it appeared that miR-127 cannot directly target Dusp1 according to the in silico target-prediction analysis. Because a previous study demonstrated that Bcl6, a master transcription regulator, can be targeted by miR-127 in some solid cancer cells (19), we wondered whether Bcl6 participated in miR-127 action in the inflammatory setting. Indeed, a robust negative correlation between the levels of miR-127 and Bcl6 was observed in LPS-stimulated macrophages (Figs. 1A, 6A). Also, a conservative seed sequence for miR-127 binding was identified in 3'UTR of Bcl6.

**FIGURE 5.** Dusp1 is responsible for miR-127-mediated regulation of JNK activity. (A and B) Western blot analysis of Dusp1 level in RAW cells that were transfected with miR-127, anti-miR-127, or their NC controls, and then stimulated with LPS for the indicated time periods. (C and D) Dusp1 level in lung tissues from the mice that were pretreated with miR-127 (miR), anti-miR-127 (anti), or negative control (NC), and then subjected to LPS challenge (1 mg/kg, intratracheally). (E) Phosphorylated and total JNK level in RAW cells that were transfected with Dusp1-siRNA or the control siRNA (left), or transfected with miR-127 along with pcDNA-Dusp1 or pcDNA (right) prior to LPS stimulation. Figures shown are representative of three independent experiments.
Further study indicated that miR-127 was able to suppress the reporter activity of the intact 3′ UTR of Bcl6, but not the one with a mutation at miR-127 binding sequence. And anti–miR-127 exhibited to preclude the degradation of Bcl6 3′ UTR and enhance the reporter activity (Fig. 6C). Consistent with this, the protein level of Bcl6 was significantly decreased by miR-127 and elevated by anti–miR-127 (Fig. 6D). Also, intratracheal administration of miR-127 caused a marked reduction of Bcl6 level in the murine lungs (Fig. 6E). Thus, the data established Bcl6 as a potential target of miR-127 in vitro as well as in vivo.

Next, we wondered whether miR-127 can regulate Dusp1 expression via the activity of Bcl6, a master transcriptional regulator. We first examined the effect of Bcl6 on Dusp1 expression in macrophages, and found that enforced expression of Bcl6 considerably increased Dusp1 expression, whereas interference of Bcl6 reduced its production (Fig. 6F). Moreover, by constructing a reporter construct flanking the 5′ region upstream of the Dusp1 gene, we confirmed that enforced Bcl6 expression in murine macrophages directly induced the transcription of Dusp1, which, however, was impaired upon Bcl6 knockdown (Fig. 6G). To further corroborate the interaction between Bcl6 and Dusp1, we constructed the plasmids with the mutation of the domain ZF3 or ZF5 of Bcl6, which was previously proved to mediate Bcl6 binding to the target gene (28, 29). The data showed that the full Dusp1-driven transcriptional activity was significantly impaired by either of the mutant constructs, suggesting that Bcl6 triggered Dusp1 expression in a DNA binding-dependent manner (Fig. 6G).

This site-specific binding of Dusp1 by Bcl6 was further confirmed by chromatin immunoprecipitation assay. In addition, miR-127 proved to decrease the binding amount of Bcl6 to Dusp1, supporting the restrained expression of Dusp1 by miR-127 we observed above (Fig. 6H). Taken together, our data indicated that, by targeting Bcl6, miR-127 mediated the regulation of Dusp1 expression, which may lead to the modulation of JNK activation and macrophage polarization. In support of this, knockdown of Bcl6 remarkably enhanced JNK activation in response to LPS stimulation, whereas restoration of Bcl6 expression abrogated miR-127–enhanced activation of JNK (Fig. 6I).

**Bcl6/Dusp1/JNK circuit is crucial for macrophage activation and polarization**

To further understand the role of the Bcl6/Dusp1 axis in miR-127–mediated polarized macrophage response, we then tested whether manipulation of Bcl6 or Dusp1 expression would affect the ability of miR-127 to shape macrophage phenotype (Fig. 7A, 7B). The result showed that introduction of Bcl6 or Dusp1 in miR-127–overexpressing cells significantly reduced the expression of IL-6 or TNF-α, but enhanced the transcription of M2-associated genes Mcr1 or Mgl2 (Fig. 7C–F). On the contrary, when Dusp1 or Bcl6 was knocked down in the macrophages with miR-127 inhibition, the results are from three independent experiments. Shown are the representative images, and data are expressed as the means ± SD. *p < 0.05, **p < 0.01 by Student t test.
the repression on the production of proinflammatory cytokines was largely abrogated and the enhanced transcription of the M2-representative genes was reversed (Fig. 7G–J). Therefore, the result, together with the observed effect of JNK kinase involved (Fig. 4G–L), established the importance of the Bcl6/Dusp1/JNK circuit in miR-127–driven programming of M1/M2 gene transcription and proposed a novel regulatory mode during macrophage development.

**Inhibition of miR-127 dampens pulmonary inflammation by skewing M2 macrophage response**

Given the importance of miR-127 antagonist in directing macrophage development toward M2 but not M1 phenotype, we wondered whether miR-127 inhibition would convey a beneficial effect in LPS-induced lung inflammation and injury. To this end, the mice were intratracheally instilled with anti–miR-127 or scramble siRNA prior to LPS challenge, and the inflammatory pathology was evaluated in the lung tissues. As shown in Fig. 8A–C, compared with the control treatment, local delivery of anti–miR-127 in LPS-challenged mice resulted in the marked alleviation of inflammatory injuries, as evidenced by less interstitial edema and debris deposit in air space, reduced lung neutrophilic inflammation, and a lower level of protein leakage in BALF. Also, the BALF level of IL-6 and TNF-α was reduced, whereas IL-10 secretion was elevated upon anti–miR-127 treatment (Fig. 8D). Furthermore, the flow cytometry analysis showed that anti–miR-127 treatment decreased the level of M1 marker, MHC II, but increased the level of M2 marker, CD206 (Fig. 8G) on AMs. In agreement with this, the transcription of inflammatory genes IL-6, TNF-α, and IL-1β was dampened, whereas expression of IL-10 and M2-characteristic genes was augmented in the AMs from the mice following miR-127 inhibition (Fig. 8F–H). The data thus demonstrated a prominent protective effect of miR-127 antagonist in the inflammatory disorders, which was largely dependent on its...
capability to shift macrophages from the proinflammatory to anti-inflammatory phenotype.

**Discussion**

In the current study, we demonstrate that miR-127 acts downstream of TLR4 to promote proinflammatory M1 macrophage development both in vitro and in vivo, which is achieved, at least partially, through a JNK-dependent mechanism. We show that TLR ligands induced a delayed, but sustained expression of miR-127, which limited Dusp1 expression via Bcl6-mediated transcriptional regulation, and thereby caused the enhanced JNK activation and proinflammatory macrophage skewing. The physiological importance of this finding is highlighted by the exaggerated pulmonary inflammation and the increased susceptibility to endotoxin shock in the mice with miR-127 treatment. Conversely, deletion of miR-127 in macrophages not only blocked JNK-mediated M1 differentiation, but also boosted the expression of the anti-inflammatory cytokine IL-10 and M2-featured genes, thereby conveying a profound protection for the mice against the inflammatory pathology (Fig. 8I). Our data thus reveal a critical role of miR-127 and the downstream signaling in the induction of pathogenic M1 macrophage activation and the transition between the pro- and anti-inflammatory phenotypes, which is believed to provide a novel insight into the molecular regulation of functional shaping of macrophages and the related inflammatory disorders.

miRNA has been thought to be a subtle and dynamic way in modulation of gene expression, and therefore has the special importance in coordinating the lineage-specific genes during macrophage development (10–12).
trigger a dynamic activation and differentiation of macrophages (37). In the current study, we found that miR-127 was transiently repressed at the early stage of the stimuli exposure and then underwent a rapid and sustained induction. This initial constraint of miR-127 was coincident with the high level of Bcl6 and Dusp1, which in turn caused the controlled activation of JNK and the restrained development of proinflammatory M1 macrophages. Nevertheless, the brake of Bcl6/Dusp1 can be effectively removed by the induction of miR-127 upon the sustained stimulation, and thereby initiates the inflammatory response. Thus, it appears that, by fine-tuning the Bcl6/Dusp1/JNK cascade, miR-127 calibrates the activation signaling for macrophages to prevent an energy-costly response under short-term stress while maintaining the dynamic responsiveness when necessary (38). Additionally, we noted that there occurred a slight decrease in miR-127 production at the late stage of the TLR response. Although the exact role of this downregulation remained to be explored, the observed M1-promoting and M2-counteracting property of miR-127 promoted us to speculate that this repressed state of miR-127 may serve as a host self-limiting mechanism to curtail the overzealous inflammatory signaling. Thus, in this study, we reveal an unexpected and fundamental role for miR-127 in coordinating the signaling required for optimal activation of macrophages and the immunological homeostasis.

Another point of our finding is the identification of the role of the Dusp1/JNK axis in molecular regulation of macrophage development. Data showed that, by titrating the relative strength of Dusp1 and JNK, miR-127 was able to modulate the lineage-defining signaling and facilitate the specification of macrophage responses. Dusp1 is an archetypal member of the dual specificity protein phosphatase family with the ability to dephosphorylate a variety of MAPKs (39). Previous studies demonstrated that, in response to bacterial infection, upregulation of Dusp1 led to deactivation of JNK and p38 (40). Our data indicated that only JNK activity among the three major MAPKs was negatively regulated by Dusp1. This discrepancy may be explained by the differential substrate specificity of MAPKs, or the differential action mode of Dusp1 in the given setting. Indeed, a conserved docking site has been identified in the sequences of ERK2, p38α, and JNK1 kinases (41). However, mutation of a cluster of functional residues within this domain only impaired the binding of Dusp1 to ERK2 and p38α, but not JNK1, suggesting a distinct interaction between Dusp1 and JNK. Remarkably, this selective modulation of JNK by Dusp1 has been evidenced in other vital biological activities. Glucocorticoids, a widely used anti-inflammatory agent, exert their effect by induction of Dusp1 via dimerization of the glucocorticoid receptor. The disruption of glucocorticoid receptor dimerization accordingly diminished Dusp1 production and caused hyperactivation of JNK, which led to the exaggerated cellular apoptosis and inflammatory injury (42). Also, in the rodent lungs, following the injurious ventilation or endotoxin, an induction of Dusp1 largely abolished the anti-inflammatory and M2-favoring effect of anti–miR-127. The data convincingly support a novel regulatory mode whereby miR-127 and the subsequent signaling of Bcl6/JNK axis in macrophages to prevent an energy-costly response under short-term stress while maintaining the dynamic responsiveness when necessary (38). Additionally, we noted that there occurred a slight decrease in miR-127 production at the late stage of the TLR response. Although the exact role of this downregulation remained to be explored, the observed M1-promoting and M2-counteracting property of miR-127 promoted us to speculate that this repressed state of miR-127 may serve as a host self-limiting mechanism to curtail the overzealous inflammatory signaling. Thus, in this study, we reveal an unexpected and fundamental role for miR-127 in coordinating the signaling required for optimal activation of macrophages and the immunological homeostasis.

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Although we reveal the modulation of Dusp1 by miR-127 in macrophage activity, it appears that miR-127 does not exert a direct effect on Dusp1 as no matching sequence has been identified within 3′ UTR of Dusp1. To further understand miR-127–initiated regulatory mode, we searched for the predicted targets and focused our attention on Bcl6, a putative target of miR-127 previously identified in human cancers. Bcl6 is generally regarded as an oncogene that has been implicated in the pathogenesis of acute leukemia and certain solid tumors (19, 45). In human breast cancers, Bcl6 proved to be one of the target genes of miR-127, and a high level of Bcl6 upon the restricted miR-127 was believed to contribute substantially to the metastasis and proliferation of carcinoma cells (19). Bcl6 also functions as a master regulator in the immune system and plays an essential role in the generation of the germinal center and development of follicular T cells (29, 46). Additionally, it has been reported that Bcl6 can inhibit a cohort of inflammatory genes by antagonizing NF-κB activity (47). However, till now there is little knowledge on how Bcl6 controls macrophage development, and the functional connection between miR-127 and Bcl6 in the inflammatory setting has never been investigated. Our current study identifies Bcl6 as a potential target of miR-127, and more importantly, as a key regulator for polarized macrophage response. miR-127–mediated modulation of Bcl6 not only impacts the TLR4-initiated inflammatory response, but also determines macrophage differentiation. This effect is achieved, at least partially, through regulating the phosphatase Dusp1 and its downstream kinase (48). Additionally, as Bcl6 is generally conceived as a transcription repressor, the finding about the direct induction of Dusp1 extends our understanding on Bcl6 action in the gene regulation.

Despite the regulatory mode we propose in this work, it has been reported that Bcl6 can negatively regulate the expression of inflammatory cytokines by counteracting NF-κB activity. We currently cannot completely exclude the direct effect of Bcl6 on macrophage responses. However, it should be noted that M1-promoting and M2-counteracting effect of miR-127 was profoundly impaired upon restoring the expression of Bcl6 or Dusp1, or blocking JNK activity. In contrast, specific depletion of Bcl6 or Dusp1 largely abolished the anti-inflammatory and M2-favoring effect of anti–miR-127. The data convincingly support a novel regulatory mode whereby miR-127 and the subsequent signaling direct the macrophage specification and the immunopathology (49, 50). Further studies may be required to determine how JNK signaling drives the lineage-specific gene transcription.

Using a prototypical model of lung injury and inflammation, we have established the significance of miR-127 regulatory loop in the pathogenesis of the inflammatory disorders. Intriguingly, a previous study indicated that miR-127 had an inhibitory effect on immune complex (IC)–induced lung inflammation and injury, presumably by downregulation of the IgFcY receptor (21). The result appears to be paradoxical to our finding. However, it has been proven that FcγR ligation on macrophages can enable macrophages to acquire a M2-like phenotype and preferentially secrete IL-10 (51, 52). In this sense, the observation that miR-127 inhibited IC-triggered inflammation may become another...
evidence to support our conclusion that miR-127 exerts a negative effect on M2 macrophage skewing. Notably, this dual regulation has been demonstrated in other immune regulators. For example, the inositol 3-phosphatase, phosphatase, and tensin homolog deleted on chromosome 10 was revealed to negatively regulate the FcyR signaling, but promoted TLR4 signaling in murine macrophages (53). Also, loss of SOCS3 profoundly decreased IC3-triggered production of inflammatory mediators, yet it boosted the endothoxin-induced lung inflammation and injury (54).

Interestingly, we also notice a reduced expression of SOCS3 in miR-127-overexpressing macrophages (our unpublished data), and its relevance to the polarized macrophage response merits further investigation.

Taken together, our present data provide a nice example of how a single miRNA modulates lineage-specific macrophage development. The discovery of miR-127 as a major player in shaping the macrophage phenotype, both in vivo and in vitro, adds it to the key mechanisms that orchestrate the protective immunity and inflammatory pathology. Modulation of miR-127 and the relevant signaling molecules may have therapeutic implication for the inflammation-related diseases.

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Disclosures

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