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MicroRNA let-7c Regulates Macrophage Polarization

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Macrophages demonstrate a high degree of plasticity, with the ability to undergo dynamic transition between different functional phenotypes, depending on microenvironmental cues (1–3). Macrophages activated by TLR ligands, such as LPS or IFN-γ, are called M1 macrophages (also referred to as classically activated macrophages) (1–4). In contrast, stimulation of macrophages with Th2 cytokines, such as IL-4 or IL-13, induces the generation of M2 type macrophages (also called alternatively activated macrophages) (1–4). Treatment of bone marrow cells with GM-CSF, termed GM-BMM, and M-CSF, termed M-BMM, lead to the generation of M1 and M2 macrophages, respectively (3, 5–11). M1 macrophages produce high levels of proinflammatory cytokines, including TNF-α, and generate increased amounts of NO through enhanced expression of inducible NO synthase (iNOS), and are critical for eradicating bacterial, viral, and fungal infections (1–4). M2 macrophages are characterized by high expression of markers of alternative activation, such as arginase-1 (Arg1) and Chitinase 3-like 3 (also called YM-1), found in inflammatory zone 1 (FIZZ1) and participate in the response to parasite infection, tissue remodeling, angiogenesis, and tumor progression (1–3, 12).

The polarization of macrophages has been the focus of numerous recent studies, particularly with regard to transcriptional regulation (1). Transcriptional factors NF-kB, AP-1, C/EBP-α, PU.1, and IFN regulatory factor 5 participate in TLR-induced M1 activation, whereas STAT6, peroxisome proliferator–activated receptor-γ, IFN regulatory factor 4, C/EBP-β, and Kruppel-like factor 4 are involved in the polarization of macrophages to the M2 phenotype (1, 3). Enzymes involved in epigenetic regulation, such as Jumonji domain containing 3 (JMJD3) and histone deacetylase 3, also play important roles in M2 macrophage polarization (3, 13, 14).

MicroRNAs (miRNAs) are a class of noncoding small RNAs, 22 nt in length, which bind to the 3’ untranslated region (UTR) of target genes, thereby inhibiting their expression through repressing mRNA translation and/or inducing degradation of target gene transcripts (15). miRNAs play essential roles in many cellular and developmental processes, including cell proliferation, apoptosis, and differentiation, as well as organ morphogenesis (15). Aberrant expression of miRNAs is closely associated with progression of pathophysiologic conditions including diabetes, cancer, tissue fibrosis, and cardiovascular disease (16–19).

The role of miRNAs in the regulation of macrophage polarization has been largely undefined (20). In this study, we found that M-BMM demonstrate greater expression of the miRNA let-7c than do GM-BMM. We found that let-7c suppresses polarization of macrophages to the M1 phenotype and enhances M2 polarization. These data suggest that the miRNA let-7c plays a role in regulating macrophage plasticity.

Materials and Methods

Generation of mouse GM-BMM and M-BMM

GM-BMM and M-BMM were derived from bone marrow cells of C57BL/6 or BALB/c mice. In brief, after lysis of RBCs, bone marrow cells were cultured in DMEM media containing 10% FBS and 20 ng/ml GM-CSF (R&D Systems) or 50 ng/ml M-CSF (R&D Systems) for 5 d to establish GM-BMM or M-BMM macrophages. The animal protocol was approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee.

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Abbreviations used in this article: Arg1, arginase-1; FIZZ1, found in inflammatory zone-1; FR-β, folate receptor β; HO-1, heme oxygenase-1; iNOS, inducible NO synthase; JMJD3, Jumonji domain containing 3; MHC-II, MHC class II; miRNA, microRNA; siRNA, small interfering RNA; TAM, tumor-associated macrophage; UTR, untranslated region.

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Quantitative real-time PCR

Probe Master Mix kit (Roche) was used for amplification of mmu-let-7c and sn0135. TaqMan probes for mmu-let-7c and sn0135 were purchased from Applied Biosystems. SYBR Green Master Mix kit (Roche) was used for the following genes. Primer sequences were: mouse GAPDH: sense, 5'-CCGACCTCTCCACACTCCCTCTC-3'; antisense, 5'-TGGCTG-GTGCCAGGTGTTCTTCTCTT-3'; mouse Arg1: sense, 5'-TGACT- GAAGTAGACAAGCTGGGGAT-3'; antisense, 5'-TGAATCTCTACACGTGTTGGAG-AG-3'; mouse Fizz1: sense, 5'-AGTTGGAAGATTTGTGTGCAGGTTAAG-3'; antisense, 5'-AACAGCTTCGTCGGGTGCTGTTCC-3'; mouse TLR4: sense, 5'-CGAGTTTTGGTCTCTTCCGTCC-3'; antisense, 5'-TTCCTGCTGTGCTGTGCTACAGTTCCG-3'; mouse CCR7: sense, 5'-CACTGTGGCATCCAGGAAACGCTCTTTACGC-3'; antisense, 5'-AAACGTGCTGGTGGTGGCTCTC-3'; mouse CD36: sense, 5'-TTTCAAACACAGGAAAGTTCCA-3'; antisense, 5'-CAGACAGAGACGCCATTCC-3'; mouse JMJD3: sense, 5'-TACACCAAACAAGG-TGGAAGAGGACTC-3'; antisense, 5'-AAATCTGACGGTTCTGAGG-3'.

Flow cytometry assay

GM-BMM cells were trypan blue-stained and suspended in PBS containing 1% BSA and 1 μg/ml Fc blocker (rat anti-mouse CD16/CD32; BD Phar-mingen) for 30 min. The cells were then incubated with 1 μg/ml FITCconjugated mouse anti-MHC class II (anti-MHC-II) mAb for 30 min. Cells were washed once and flow cytometry was performed.

Transfection of miRNAs and small interfering RNAs

GM-BMM and M-BMM were transfected with 20 nM miRNA mimics, 20 nM miRNA inhibitors, or 20 nM small interfering RNAs (siRNAs) using HiperFect transfection reagent (Qiagen) according to the manufacturer’s instructions. Control and let-7c mimics were from Life Technologies. Control inhibitors and inhibitors against let-7c were from Exiqon. Control siRNA and C/E/BP-8 siRNA were from Dharmacon.

Experimental pulmonary fibrosis model

Bleomycin-induced mouse pulmonary fibrosis model was established as previously described by our group (24). Eight-week-old male C57BL/6 mice were used in this study. Alveolar macrophages were obtained from bronchoalveolar lavage. In brief, cells from the bronchoalveolar lavage were plated in 24-well plates for 30 min. The plates were then washed extensively to remove unattached cells. Attached macrophages were lysed and RNA isolated.

Statistical analysis

One-way ANOVA followed by the Bonferroni test was performed for multiple-group comparisons. The Student t test was used for comparison between two groups. A p value <0.05 was considered statistically signif-

Results

M-BMM demonstrate greater expression of let-7c than do GM-BMM

To study macrophage polarization and plasticity, we chose GM-BMM and M-BMM as macrophages representative of the two opposite polarized states (M1 versus M2) as their phenotypes have been well defined in numerous studies (5–11). We found that M-BMM exhibit a considerably higher level of let-7c than do GM-BMM (Fig. 1A). Genetic background of mice had no effect on this phenomenon because let-7c also demonstrated significantly greater levels in M-BMM than in GM-BMM that were derived from BALB/c mice (Supplemental Fig. 1). Our initial findings suggest that let-7c participates in macrophage polarization.

As TLR4 stimulation promotes and induces M1 polarization (4), we next asked whether TLR4 stimulation affects let-7 levels in M-BMM. We found that let-7c levels are significantly reduced in M-BMM (Fig. 1A). Genetic background of mice had no effect on this phenomenon because let-7c also demonstrated significantly greater levels in M-BMM than in GM-BMM that were derived from BALB/c mice (Supplemental Fig. 1). Our initial findings suggest that let-7c participates in macrophage polarization.

To examine whether let-7c contributes to the plasticity of macrophage polarization, we attempted to convert one population into another by culturing GM-BMM macrophages with M-CSF and M-BMM macrophages with GM-CSF. As shown in Fig. 1C, GM-BMM–to–M-BMM conversion resulted in increased let-7c, whereas M-BMM–to–GM-BMM conversion led to decreased let-7c expression. Of note, the alteration in let-7c levels in the converted GM-BMM or M-BMM was less than that between GM-BMM and M-BMM. These data suggest that the conversion by culture of GM-BMM or M-BMM with M-CSF or GM-CSF is incomplete.

To determine whether the increase in let-7c during GM-BMM–to–M-BMM conversion occurs in pathologic conditions where M2 macrophages play important roles (25–28), we examined let-7c levels in alveolar macrophages isolated from fibrotic mouse lungs. These macrophages are known to express M2 phenotypes (25–28). We found that let-7c is upregulated in alveolar macrophages isolated from fibrotic mouse lungs, compared with macrophages from...
normal mouse lungs (Fig. 1D). As expected, the M2 macrophage marker, Arg1, and JMJD3, a histone demethylase that was previously shown to have higher levels in M2 macrophages (6), were increased in alveolar macrophages isolated from fibrotic mouse lungs (Fig. 1D). Given the established role of M2 macrophages in lung remodeling and fibrosis (25–28), these data suggest that let-7c may participate in pulmonary fibrosis through modulating alveolar macrophage polarization. Further studies on the effect of let-7c–regulated alveolar macrophages isolated from fibrotic mouse lungs (Fig. 1D) may provide insight into this hypothesis. Homogenous staining of let-7c–regulated alveolar macrophages on pulmonary fibrosis will likely provide insight into this hypothesis.

Overexpression of let-7c diminishes the expression of M1 phenotypes in GM-BMM

To determine whether let-7c participates in macrophage polarization, we transfected GM-BMM, which have lower levels of let-7c than do M-BMM, with mimics for let-7c. We found that overexpression of let-7c in GM-BMM diminishes the basal levels of CCR7 (Fig. 2A), a typical marker of GM-BMM (30). These data suggest that let-7c is a negative regulator of M1 macrophage phenotypes.

TLR4 stimulation promotes M1 macrophage polarization, as characterized by enhanced expression of proinflammatory cytokines, such as IL-12, and iNOS (4, 7, 31). To investigate the effect of let-7c on the proinflammatory response of macrophages, we treated GM-BMM transfected with control mimics or mimics for let-7c with LPS. As shown in Fig. 2B, the increases in IL-12 and iNOS expression normally found after LPS treatment were diminished in let-7c transfected GM-BMM. Furthermore, let-7c attenuated LPS-enhanced MHC-II levels on the surface of GM-BMM. These data suggest that let-7c is a negative regulator of proinflammatory responses induced by TLR4 stimulation in GM-BMM. Of note, the moderate effect of let-7c on the suppression of the M1 phenotypes suggests that it may be just one of the miRNAs that are involved in this process.
**Overexpression of let-7c promotes GM-BMM transition to the M2 phenotype**

Because our experiments found that overexpression of let-7c in GM-BMM diminishes their expression of M1 phenotypes after LPS exposure, we next asked whether let-7c also participates in macrophage plasticity by promoting the transition of GM-BMM to the M-BMM or M2 phenotype. To address this question, we transfected GM-BMM with control mimics or mimics for let-7c and then first examined the expression of a typical M-BMM marker, FR-β (34). As shown in Fig. 3A, GM-BMM transfected with let-7c demonstrated increased levels of FR-β, compared with GM-BMM transfected with control mimics. These data suggest that let-7c can drive the transition of GM-BMM toward the M-BMM phenotype.

Because IL-4 is a classical Th2 cytokine that induces M2 macrophage polarization (4), we evaluated the effect of let-7c on IL-4-induced M2 polarization. In these experiments, we treated GM-BMM that were transfected with control mimics or mimics for let-7c with IL-4. We found that IL-4 induced expression of Arg1, FIZZ1, and YM-1 in GM-BMM transfected with let-7c is significantly greater than that in GM-BMM cells transfected with control mimics (Fig. 3B). Taken together, these data suggest that let-7c promotes the transition of GM-BMM to the M2 phenotype.

A previous report showed that let-7c enhances heme oxygenase-1 (HO-1) expression (35). Because HO-1 has been shown to be involved in macrophage polarization (36), we determined whether HO-1 affects let-7c-regulated M2 polarization. We treated let-7c mimics transfected GM-BMM with specific HO-1 inhibitors, zinc protoporphyrin, before IL-4 exposure. As shown in Supplemental Fig. 3, let-7c enhanced IL-4–induced expression of M2 marker Fizz1. The enhanced expression of IL-4–induced Fizz1 was diminished when HO-1 was inhibited.

**Knockdown of let-7c promotes transition of M-BMM to the M1 phenotype and diminishes the expression of M2 phenotypes in M-BMM**

We have shown that let-7c suppresses expression of the M1 phenotype and promotes transition to the M2 phenotype in GM-BMM. We next asked whether knocking down let-7c in M-BMM, which have higher levels of let-7c than do GM-BMM, demonstrates an effect opposite to that observed in GM-BMM that are transfected with let-7c. To answer this question, we transfected M-BMM with control inhibitors or inhibitors against let-7c. As shown in Fig. 4A, let-7c knockdown enhanced LPS-induced expression of IL-12 and iNOS. Given our findings that GM-BMM with overexpression of let-7c have diminished proinflammatory response to LPS, these data establish that let-7c has a suppressive role in M1 macrophage polarization.

**Knockdown of let-7c promotes transition of M-BMM to the M1 phenotype and diminishes the expression of M2 phenotypes in M-BMM**

Because our experiments demonstrated that knockdown of let-7c promotes M-BMM transition to M1 phenotype, we next asked whether knockdown of let-7c has any effect on expression of the M2 phenotype. As shown in Fig. 4B, knockdown of let-7c diminished the levels of FR-β. Furthermore, knockdown of let-7c diminished IL-4–induced progression of M-BMM to the M2 phenotype, as shown by a decrease in IL-4–induced Arg1, FIZZ1, and YM-1 in these cells (Fig. 4C). These data suggest that let-7c participates in sustaining the M2 macrophage phenotype.

**let-7c does not affect signaling events that are immediately downstream of the engagement of LPS or IL-4 with their receptors**

LPS binding to TLR4 induces IκB-α degradation, which leads to NF-κB activation (37). In addition, LPS stimulation activates MAPKs, such Erk and p38 (37). Activation of NF-κB and MAPKs is required for the proinflammatory responses that occur in macrophages after LPS stimulation (37). To determine whether let-7c inhibits signaling events that are immediately downstream of engagement of TLR4 by LPS, we examined IκB-α degradation as well as phosphorylation of Erk and p38 in LPS-treated GM-BMM that were transfected with control mimics or mimics for let-7c. As shown in Supplemental Fig. 4A, overexpression of let-7c had no effect on LPS-induced IκB-α degradation or phosphorylation of Erk and p38 in GM-BMM. Knockdown of let-7c in M-BMM also did not affect LPS-induced IκB-α degradation or phosphorylation of Erk or p38 (Supplemental Fig. 4C). These data suggest that inhibition of macrophage transition to the M1 phenotype by let-7c is not caused by alterations in cytoplasmic transduction of TLR4 signaling.
IL-4 stimulation induces STAT6 phosphorylation and translocation to the nucleus (6, 38). Activation of STAT6 is required for IL-4–induced polarization of macrophages to the M2 phenotype (6, 38). To determine whether let-7c enhances STAT6 activation by IL-4, thereby augmenting IL-4–induced M2 macrophage polarization, we examined STAT6 phosphorylation in IL-4–treated GM-BMM that were transfected with control mimics or mimics for let-7c. As shown in Supplemental Fig. 4B, overexpression of let-7c had no effect on IL-4–induced STAT6 phosphorylation in GM-BMM. Knockdown of let-7c in M-BMM also did not affect IL-4–induced STAT6 phosphorylation (Supplemental Fig. 4D). These data suggest that the promotion of development of the M2 phenotype by let-7c is not caused by alterations in IL-4–induced STAT6 activation.

**let-7c targets C/EBP-δ**

To delineate the mechanism by which let-7c regulates macrophage polarization, we searched predicted targets of let-7c that could participate in this process and found that the transcriptional factor C/EBP-δ is among let-7c targets. C/EBP-δ has been shown to regulate proinflammatory response to LPS (39–41). To determine whether let-7c targets C/EBP-δ in macrophages, we transfected GM-BMM with control mimics or mimics for let-7c. As shown in Fig. 5A and 5B, overexpression of let-7c diminished both mRNA and protein expression of C/EBP-δ. Next, we cloned the 3′ UTR of C/EBP-δ into a luciferase reporter and cotransfected it with control or let-7c mimics. As shown in Fig. 5C, let-7c downregulated luciferase activity of the reporter that contained the 3′ UTR of C/EBP-δ, suggesting that let-7c directly targets C/EBP-δ. To determine whether knockdown of C/EBP-δ recapitulates the suppressive effect of let-7c on the development of the M1 phenotype, we transfected GM-BMM with control siRNA or C/EBP-δ siRNA and found that C/EBP-δ knockdown attenuates LPS-induced expression of IL-12 and iNOS (Fig. 5D). In addition, C/EBP-δ knockdown diminished the levels of MHC-II in untreated GM-BMM (Fig. 5E). C/EBP-δ knockdown also attenuated LPS-enhanced MHC-II levels on the macrophage surface (Fig. 5E). In addition, C/EBP-δ knockdown diminished the levels of CCR7 in GM-BMM (Fig. 5F). In contrast, knockdown of C/EBP-δ enhanced the expression of M2 phenotype, as indicated by increased levels of FR-β (Fig. 5F). As expected, C/EBP-δ siRNA reduced the expression of C/EBP-δ in GM-BMM (Fig. 5G). These data confirmed previous findings that C/EBP-δ is a negative regulator of the proinflammatory response to TLR4 stimulation (39–41). These data also suggest that C/EBP-δ contributes, at least in part, to the effect of let-7c on macrophage polarization.

**let-7c regulates cellular functions associated with M1 and M2 phenotypes**

We have shown that let-7c suppresses M1 macrophage polarization and promotes M2 macrophage activation. We next asked whether let-7c regulates cellular functions associated with the M1 and M2 phenotypes. It was previously shown that M2 macrophages possess greater activity to engulf apoptotic cells than do M1 macrophages (42–45), which we confirmed in Fig. 6A. Next, we evaluated the effect of let-7c on the engulfment of apoptotic thymocytes by GM-BMM and M-BMM. As shown in Fig. 6B, overexpression of let-7c in GM-BMM significantly enhanced their ability to uptake apoptotic cells. In contrast, knockdown of let-7c diminished the ability of M-BMM cells to engulf apoptotic thymocytes (Fig. 6C). The effect of let-7c on the activity of macrophages to engulf ap-
Optotic thymocytes may be caused by alterations of the levels of CD36, a receptor that mediates clearance of apoptotic cells (46), on the surface of let-7c–modulated macrophages (Fig. 6D, 6E). These data are consistent with our findings that overexpression of let-7c in GM-BMM promotes their transition to the M2 phenotype and knockdown of let-7c in M-BMM promotes their transition to the M1 phenotype.

M1 macrophages are known to have high bactericidal activity (1, 2, 4). To determine whether let-7c regulates bactericidal activity, we transfected GM-BMM with control or let-7c mimics. We found that overexpression of let-7c significantly diminished the bactericidal activity of GM-BMM (Fig. 6F). These data suggest that let-7c suppresses macrophage functions associated with the M1 phenotype.

Discussion
In these experiments, we found that let-7c promotes M2 macrophage polarization and suppresses M1 polarization. However, let-7c appeared to have no effect on the cytoplasmic signaling events that are downstream of the engagement of LPS or IL-4 with their receptors, including IκB-α degradation, MAPK activation, or STAT6 phosphorylation (38, 47). These findings indicate that let-7c may regulate TLR4 and IL-4 signaling through interactions with nuclear targets. Consistent with this role for let-7c, we found that let-7c regulates C/EBP-δ, an important transcriptional factor that has been shown to be required for a sustained TLR4-induced inflammatory response (40). Multiple lines of evidence in our study support a direct regulation of C/EBP-δ by let-7c, including 1. C/EBP-δ is a key transcription factor in macrophage polarization, and its expression is regulated by let-7c. 2. C/EBP-δ contains a binding site for let-7c in its 3' UTR. 3. Overexpression of let-7c downregulates C/EBP-δ in GM-BMM at both mRNA and protein levels. 4. C/EBP-δ 3' UTR is responsive to let-7c regulation.

Our data suggest that C/EBP-δ may be involved in mediating the effects of let-7c on macrophage polarization. This supposition is based on our findings that knocking down C/EBP-δ diminishes M1 macrophage activation whereas enhancing M2 polarization.
Although the role of C/EBP-δ in M1 activation has been studied (39–41), it is unknown how C/EBP-δ regulates M2 macrophage polarization. It is possible that C/EBP-δ may modulate STAT6 binding to the promoters of the M2 macrophage marker genes, an action similar to its regulation of NF-κB in cellular responses that promote M1 activation (40). It is also likely that C/EBP-δ may interfere with C/EBP-β in inducing the M2 marker genes. C/EBP-β has been shown to be required for the expression of M2 macrophage phenotype (48). However, C/EBP-δ may not be the sole mediator of let-7c in regulating macrophage polarization because knockdown of C/EBP-δ does not duplicate all of the effects of let-7c in macrophages. Indeed, recent studies found that let-7 family members repress the expression of TLR4 and cytokine-inducible Src homology 2–containing protein in cholangiocytes and contribute to epithelial immune responses against Cryptosporidium parvum infection (49, 50).

We found that let-7c is expressed at higher levels in alveolar macrophages from fibrotic mouse lungs than in alveolar macrophages from normal mouse lungs. These data are concordant with previous findings that alveolar macrophages in fibrotic lungs possess the M2 macrophage phenotype (25–28). One of the main functions of M2 macrophages is to promote wound repair. M2 macrophages are also implicated in fibrotic diseases that feature uncontrolled, excessive collagen deposition and production of extracellular matrix proteins. Therefore, targeting let-7c in alveolar macrophages may have potential utility in the treatment of pulmonary fibrosis.

Our experiments demonstrated that let-7c not only regulates the expression of M1 and M2 macrophage markers, but also controls macrophage functions associated with the M1 and M2 states. We found that overexpression of let-7c in GM-CSF diminished their activity to kill bacteria, a typical function of M1 macrophages (1, 2, 4). We also found that overexpression of let-7c in GM-BMM enhanced their ability to engulf apoptotic cells, an event that has been implicated in M2 macrophage polarization (42–45). Concordantly, knockdown of let-7c in M-BMM diminished their capability to engulf apoptotic cells. All of these data suggest that let-7c has a role in controlling the plasticity of macrophage differentiation. Therefore, any dysregulation of let-7c could impair the ability of macrophages to rapidly switch differentiation state, which is required for macrophages to mount an appropriate response to environmental cues. Conceivably, a failure of appropriate macrophage polarization will lead to pathologic conditions.

Let-7 is one of the first miRNAs identified and initially was found to control developmental timing in Caeorhabditis elegans (51, 52). Members of let-7 family have been shown to either promote or inhibit inflammatory response to various stimuli (50, 53–58). The differential regulation might be because of different cell populations and stimuli examined. A number of studies also found that let-7 is frequently downregulated in cancers, and that the loss of the tumor-suppressor activity of let-7 is associated with worse outcomes in cancer patients (59). Recently, tumor-associated macrophages (TAMs) have been shown to have a major role in the regulation of tumor progression by controlling angiogenesis and immune suppression (12). TAMs have been shown to share some of the characteristics of the M2 macrophage phenotype (12). Levels of let-7 family members in TAMs are currently unknown. Although our experiments showed that let-7c promotes M2 polarization and suppressed M1 activation, it is presently not possible to infer that reduced expression of let-7c in TAMs, as is true in various cancer cells (59), may diminish their degree of M2 polarization.

In these studies, we found that M-BMM demonstrate greater expression of let-7c than do GM-BMM. Furthermore, we demonstrated that when M-BMM were converted to GM-BMM by being cultured in GM-CSF or vice versa, the levels of let-7c also reversed. Although the mechanisms by which let-7c levels are regulated during the transition between the M1 and M2 polarization remains to be determined, our data suggest that dynamic changes in the expression of let-7c, and likely other miRNAs (20, 33, 60), may contribute to an innate mechanism that may be used by macrophages to respond effectively to environmental cues.

Disclosures

The authors have no financial conflicts of interest.

References


