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microRNA let-7c regulates macrophage polarization

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Abstract

Macrophages demonstrate a high level of plasticity, with the ability to undergo dynamic transition between M1 and M2 polarized phenotypes. The role of miRNAs in regulating macrophage polarization has been largely undefined. In this study, we found that microRNA let-7c is expressed at a higher level in M-BMM (M2 macrophages) than in GM-BMM (M1 macrophages). let-7c levels are also greater in alveolar macrophages from fibrotic lungs as compared to those from normal lungs. let-7c expression was decreased when M-BMM converted to GM-BMM whereas increased when GM-BMM converted to M-BMM. LPS stimulation reduced let-7c expression in M-BMM. We found that overexpression of let-7c in GM-BMM diminished M1 phenotype expression while promoting polarization to the M2 phenotype. In contrast, knockdown of let-7c in M-BMM promoted M1 polarization, and diminished M2 phenotype expression. We found that let-7c targets C/EBP-δ, a transcriptional factor that plays an important role in inflammatory response. Furthermore, we found that let-7c regulates bactericidal and phagocytic activities of macrophages, two functional phenotypes implicated in macrophage polarization. Our data suggest that the microRNA let-7c plays an important role in regulating macrophage polarization.

Keywords

microRNA let-7c; M1 macrophage; M2 macrophage; C/EBP-δ

Introduction

Macrophages are derived from hematopoietic stem cells through bone marrow myeloid progenitor cells, and show a high degree of plasticity, with the ability to undergo dynamic transition between different functional phenotypes, depending on micro-environmental cues (1–3). Macrophages activated by toll like receptor (TLR) ligands, such as lipopolysaccharide (LPS), or IFN-γ, are called M1 macrophages (also referred to as classically activated macrophages) (1–4). On the other hand, 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 granulocyte macrophage-colony stimulation factor (GM-CSF), termed GM-BMM, and macrophage-colony stimulation factor (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 nitric oxide (NO) through enhanced expression of inducible NO synthase (iNOS), and are critical

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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), 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-κB, AP-1, CCAAT/enhancer-binding protein α (C/EBP-α), PU.1 and IFN-regulatory factor 5 (IRF5) participate in TLR induced M1 activation, whereas signal transducer and activator of transcription 6 (STAT6), peroxisome proliferator-activated receptor (PPAR)-γ, IRF4, C/EBP-β, and Kruppel-like factor 4 (KLF4) 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 (HDAC3), also play important roles in M2 macrophage polarization (3, 13, 14).

microRNAs (miRNAs) are a class of non-coding small RNAs, 22 nt. in length, which bind to the 3’ 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 microRNA let-7c plays a role in regulating macrophage plasticity.

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. Briefly, after lysis of red blood cells, 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 days to establish GM-BMM or M-BMM macrophages. The animal protocol was approved by the UAB Institutional Animal Care and Use Committee (IACUC).

Quantitative real-time PCR

Probe Master Mix kit (Roche) was used for amplification of mmu-let-7c and sno135. Taqman probes for mmu-let-7c and sno135 were purchased from Applied Biosystems. SYBR Green Master Mix kit (Roche) was used for the following genes. Primer sequences were: mouse GAPDH: sense, 5' CGACTTCAACAGCAACTCCCACTCTTCC 3'; antisense, 5' TGGGTGGTCCAGGGTTTCTTACTCCTT 3'; mouse Arginase-1: sense, 5' TGACTGAAGTAGACAAGCTGGGGAT 3'; antisense, 5' CGACATCAAAGCTCAGGTGAATCGG; mouse YM-1: sense, 5' ATGAAGCATTGAATGGTCTGAAAG 3'; antisense, 5' TGAATATCTGACGGTTCTGAGGAG 3'; mouse FIZZ1: sense, 5' AGGTCAAGGAACTTCTTGCCAATCC 3'; antisense, 5' AAGCACACCCAGTAGCAGTCATCCC 3'; mouse iNOS: sense, 5' ATCTTTGCCACCAAGATGGCCTGG 3'; antisense, 5' TTCCTGTGCTGTGCTACAGTTCCG 3'; mouse C/EBP-δ; sense 5'

AACTTGATTCCTCGTTGCCTCTACTTTC 3'; antisense, 5' CCGCAAACATTACAATTACTGGCTTTT 3'; mouse JMJD3: sense, 5' TACCCCCAGCATCTATTTGGAGAGC 3'; antisense, 5' TAAGTTGAGCCGAAGTGAACCAGCC 3'; mouse IL-12 p40: sense, 5' CCAAATTACTCCGGACGGTTCAC 3'; antisense, 5' CAGACAGAGACGCCATTCCACAT 3'; mouse FR-β: sense, 5' CTTCAACTGGGATCACTGTGGCA 3'; antisense, 5' CATCCAGGAAACGCTCTTTACGC 3'; mouse CCR7: sense, 5' AAACGTGCTGGTGGTGGCTCTC 3'; antisense, 5' ACCGTGGTATTCTCGCCGATGT 3'; mouse CD36: sense, 5' TTTCAATGGAAAGGATAACATAAGCAAAGT 3'; antisense, 5' TCAACAAAAGGTGGAAAGGAGGCT 3'.

Western blotting

Western blotting was performed as previously described (21). Mouse anti-actin antibody, anti-GAPDH and rabbit anti-C/EBP-δ antibodies were from Santa Cruz. Rabbit anti-IκB-α, rabbit anti-pp-38, rabbit anti-p-Erk, and rabbit anti-p-STAT6 antibodies were from Cell Signaling.

Luciferase assay

cDNA containing the full-length C/EBP-δ 3' UTR sequence was obtained by PCR amplification using a human C/EBP-δ EST (Open Biosystems) as a template and cloned into SpeI and HindIII sites of pMir-Report Luciferase vector (Applied Biosystems). The sequences of the primers used for the PCR amplification were: sense: 5' GGACTAGTCAGTTCTTCAAGCAGCTGCC 3' and antisense: 5' CCAAGCTTGTGAGCATGCTCAGTCTTTTCC 3'. The resulting construct was designated as pMir-Report-C/EBP-δ. HEK-293T cells were transfected with 5 ng pMir-Report-C/EBP-δ and 20 nM control mimics or 20 nM mimics for let-7c using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. After 72 hours of transfection luciferase activity in the cells was determined using a Luciferase Assay System (Promega).

Bacteria killing assay

Bacterial killing capability of BMM was performed as described previously (22). 0.1×10^6 CFU/ml *E.coli* (BL21DE3pLysS) were added to GM-BMM or M-BMM in 96-well plates. Plates were centrifuged for 5 minutes at 400*g* and incubated for 1 hour at 37 °C. Supernatants from each well were subject to 100-fold dilutions and 100 μl of the diluted supernatants was plated on Luria broth (LB)-agar plates. The plates were incubated at 37°C over night and bacterial colonies enumerated. Data was presented as CFU/ml. CFU/ml= numbers of bacterial colonies×dilution factor/volume the diluted supernatants plated.

Phagocytosis assay

Phagocytosis assay was performed as previously described by our group (23). Briefly, phagocytosis of PKH26-labeled apoptotic thymocytes by GM-BMM or M-BMM (efferocytosis) was evaluated by addition of 0.5×10^6 apoptotic thymocytes suspended in 500 μl of RPMI 1640 media containing 10% FBS to the cells, followed by incubation at 37°C for 30 min. Macrophages were then washed 5 times with cold PBS, resuspended in PBS containing 1% albumin, FITC-conjugated anti-CD11b (macrophage marker) antibody, and APC-conjugated anti-CD90.2 antibody (thymocyte marker), and analyzed by flow cytometry. The phagocytic index was calculated as the ratio of $FITC^+PKH26^+APC^-$ cells to all cells gated. Engulfed thymocytes are not accessible to the APC-conjugated anti-CD90.2 antibody. Therefore, $FITC^+PKH26^+APC^-$ cells are macrophages that have engulfed PKH-labeled thymocytes. For Fc receptor mediated phagocytosis, red fluorescent labeled

carboxylate-modified beads (Invitrogen) were pre-incubated with mouse IgG (5 mg/ml) for 1h. The beads were then incubated with macrophages for 15 minutes. The cells were washed 3 times and cell suspension prepared. Flow cytometry assays were performed.

Flow cytometry assay

GM-BMM cells were trypsinized and suspended in PBS containing 1% BSA and 1 μg/ml Fcγ blocker (rat anti-mouse CD16/CD32, BD Pharmingen) for 30 min. The cells were then incubated with 1 μg/ml FITC conjugated mouse anti-MHCII monoclonal antibody for 30 min. Cells were washed once and flow cytometry was performed.

Transfection of miRNAs and siRNAs

GM-BMM and M-BMM were transfected with 20 nM miRNA mimics, 20 nM miRNA inhibitors or 20 nM 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/EBP-δ siRNA were from Dharmacon.

Experimental pulmonary fibrosis model

Bleomycin induced mouse pulmonary fibrosis model was established as previously described by our group (24). 8-week male C57BL/6 mice were used in this study. Alveolar macrophages were obtained through bronchoalveolar lavage (BAL). Briefly, cells from the BAL 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. $P < 0.05$ was considered statistically significant.

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 vs. 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 (Figure 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 (Supplementary Figure 1), Our initial findings suggest that let-7c participates in macrophage polarization.

As TLR4 stimulation and promotes induces M1 polarization (4), we next asked if TLR4 stimulation affects let-7 levels in M-BMM. We found that let-7c levels are significantly reduced in LPS treated M-BMM (Figure 1B). These data indicate that a decrease in let-7c may be involved in the promotion of M1 phenotype expression.

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 Figure 1C, GM-BMM-to-M-BMM conversion resulted in increased let-7c, whereas M-BMM-to-GM-BMM 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 if the increase in let-7c during GM-BMM-to-M-BMM conversion occurs in pathological 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 macrophages from normal mouse lungs (Figure 1D). As expected, the M2 macrophage marker, arginase 1 (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 (Figure 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 on pulmonary fibrosis will likely provide insight into this hypothesis. Homogenous staining of CD11c, a specific surface marker of alveolar macrophages (29), indicates the purity of these cells (Supplementary Figure 2).

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

To determine if 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 (Figure 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 pro-inflammatory cytokines, such as IL-12, and iNOS (4, 7, 31). To investigate the effect of let-7c on the pro-inflammatory response of macrophages, we treated GM-BMM transfected with control mimics or mimics for let-7c with LPS. As shown in Figure 2B, the increases in IL-12 and iNOS expression normally found after LPS treatment were diminished in let-7c transfected GM-BMM. Additionally, we examined MHCII expression, one of the surface markers of M1 macrophages (32, 33), in GM-BMM transfected with control mimics or mimics for let-7c. As shown in Figures 2C and 2D, transfection with let-7c diminished levels of MHCII in GM-BMM. Furthermore, let-7c attenuated LPS enhanced MHCII levels on the surface of GM-BMM. These data suggest that let-7c is a negative regulator of pro-inflammatory 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

As our experiments found that overexpression of let-7c in GM-BMM diminishes their expression of M1 phenotypes after LPS exposure, we next asked if 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, folate receptor β (FR-β) (34). As shown in Figure 3A, GM-BMM transfected with let-7c demonstrated increased levels of FR-β, compared to GM-BMM transfected with control mimics. These data suggest that let-7c can drive the transition of GM-BMM toward the M-BMM phenotype.

As 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 (Figure 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). As HO-1 has been shown to be involved in macrophage polarization (36), we determined if HO-1 affects let-7c regulated M2 polarization. We treated let-7c mimics transfected GM-BMM with specific HO-1 inhibitors, zinc protoporphyrin (ZnPP), before IL-4 exposure. As shown in Supplementary Figure 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 if 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 Figure 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 pro-inflammatory response to LPS, these data establishes that let-7c has a suppressive role in M1 macrophage polarization.

As our experiments demonstrated that knockdown of let-7c promotes M-BMM transition to M1 phenotype, we next asked if knockdown of let-7c has any effect on expression of the M2 phenotype. As shown in Figure 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 (Figure 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). Additionally, LPS stimulation activates MAPKs, such Erk and p38 (37). Activation of NF-κB and MAPKs are required for the pro-inflammatory responses that occur in macrophages following LPS stimulation (37). To determine if 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 Supplementary Figure 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 (Supplementary Figure 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 if 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 Supplementary Figure 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 (Supplementary Figure 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 pro-inflammatory response to LPS (39–41). To determine if let-7c targets C/EBP-δ in macrophages, we transfected GM-BMM with control mimics or mimics for let-7c. As shown in Figures 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 co-transfected it with control or let-7c mimics. As shown in Figure 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 if 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 (Figure 5D). Additionally, C/EBP-δ knockdown diminished the levels of MHCII in untreated GM-BMM (Figure 5E). C/EBP-δ knockdown also attenuated LPS enhanced MHCII levels on the macrophage surface (Figure 5E). Additionally, C/EBP-δ knockdown diminished the levels of CCR7 in GM-BMM (Figure 5F). In contrast, knockdown of C/EBP-δ enhanced the expression of M2 phenotype, as indicated by increased levels of FR-β (Figure 5F). As expected, C/EBP-δ siRNA reduced the expression of C/EBP-δ in GM-BMM (Figure 5G). These data confirmed previous findings that C/EBP-δ is a negative regulator of the pro-inflammatory 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 if 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 Figure 6A. Next, we evaluated the effect of let-7c on the engulfment of apoptotic thymocytes by GM-BMM and M-BMM. As shown in Figure 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 (Figure 6C). The effect of let-7c on the activity of macrophages to engulf apoptotic thymocytes may be caused by alterations of the levels of CD36, a receptor that mediate clearance of apoptotic cells (46), on the surface of let-7c modulated macrophages (Figure 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 if 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 (Figure 6F). These data suggest that let-7c suppresses macrophage functions associated with the M1 phenotype.

Figure 6G–H showed that overexpression of let-7c decreased, whereas knockdown of let-7c enhanced Fc receptor-mediated phagocytosis. Given that enhanced Fc receptor-mediated phagocytosis is a phenotype of inflammatorily activated macrophages, these data are consistent with the findings that let-7c attenuated M1 activation of macrophages.

Discussion

In the present 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 interaction 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: first, overexpression of let-7c downregulates C/EBP-δ in GM-BMM at both mRNA and protein levels; second, the 3' UTR in C/EBP-δ transcripts contains a let-7c binding site; and thirdly, the 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 while 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 since 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 (CIS) in cholangiocytes and contributes 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-BMM 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 pathological conditions.

Let-7 is one of the first miRNAs identified and initially was found to control developmental timing in *Caenorhabditis 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 due to 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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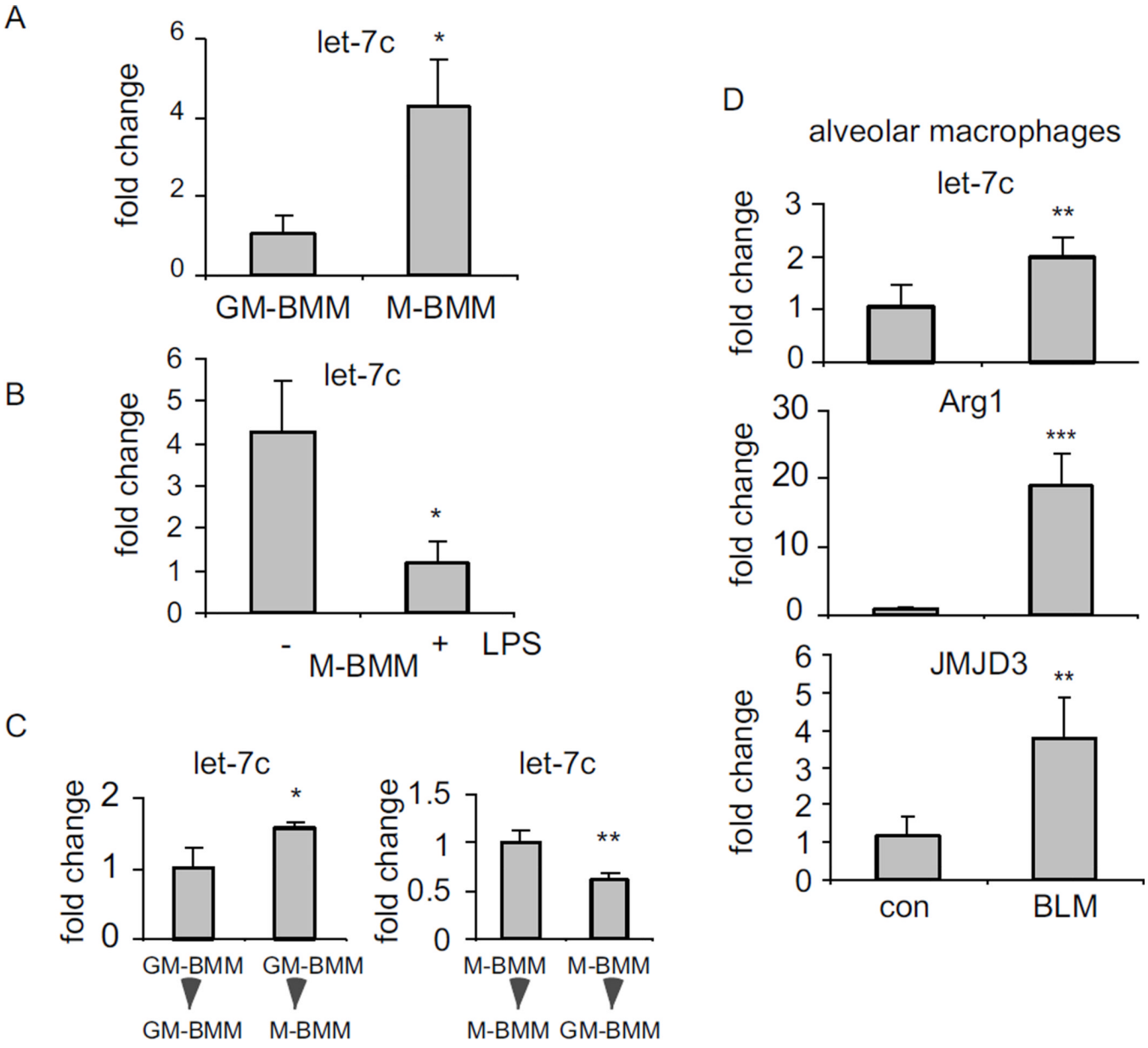

Figure 1. M-BMM demonstrate greater expression of let-7c than do GM-BMM

(A) Mouse bone marrow cells were cultured in 50 μg/ml M-CSF or 20 μg/ml GM-CSF for 7 days to establish M-BMM and GM-BMM. RNA was isolated and levels of let-7c determined by real-time PCR. Small nucleolar RNA, sno135, was used as an internal control. n=3; mean±SD; * P<0.05 compared to GM-BMM. (B) M-BMM were treated without or with 100 ng/ml LPS for 24h. RNA was isolated and levels of let-7c determined. n=3; mean±SD; * P<0.05 compared to untreated GM-BMM. (C) M-BMM and GM-BMM were established as in A. The cells were then cultured in fresh media containing GM-CSF or M-CSF for 5 more days to induce the transition from M-BMM to GM-BMM or vice versa. RNA was isolated and levels of let-7c determined. n=3; mean±SD; * P<0.05, ** P<0.01 compared to the control groups. (D) Alveolar macrophages were isolated from lungs of normal mice or bleomycin treated mice, as described in "Materials and Methods". RNA was isolated and levels of let-7c, Arg1, and JMJD3 determined. n=5 for each group; mean±SD;

** $P<0.01$, *** $P<0.001$ compared to the control group. The experiments were performed two to three times with similar results.

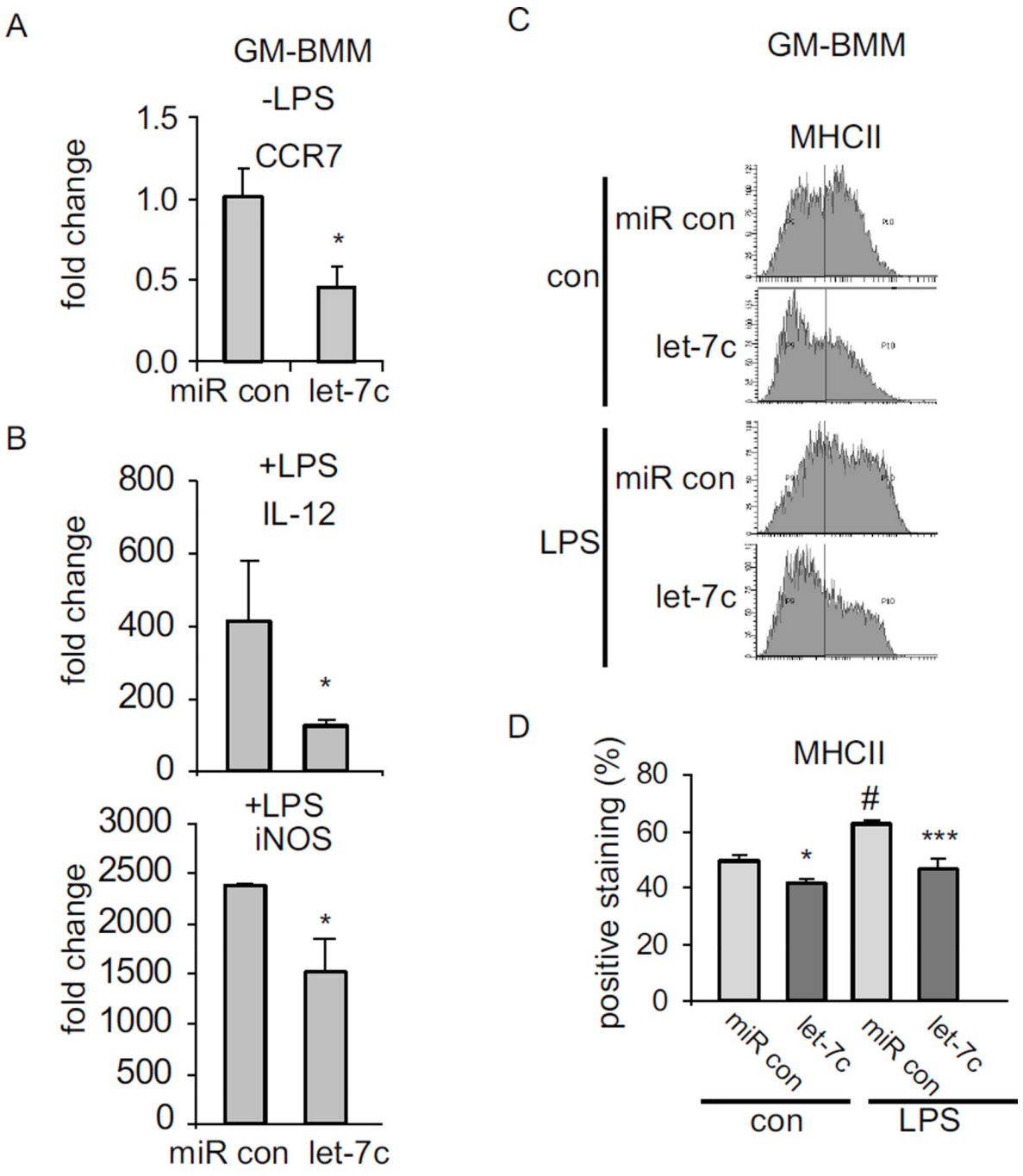

Figure 2. Overexpression of let-7c diminishes the expression of M1 phenotypes in GM-BMM

(A) GM-BMM were transfected with 20 nM control mimics or mimics for let-7c. 3 days after transfection, cells were harvested and RNA isolated. Levels of CCR7 were determined. n=3; mean±SD; * P<0.05 compared to the control group. (B) GM-BMM were transfected with 20 nM control mimics or mimics for let-7c. 3 days after transfection, the cells were treated with 100 ng/ml LPS for 6h. Levels of IL-12 and iNOS were determined. n=3; mean ±SD; * P<0.05 compared to the control groups. (C) GM-BMM were transfected with 20 nM control mimics or mimics for let-7c. 3 days after transfection, the cells were treated without or with 100 ng/ml LPS for 6h. The surface levels of MHCII were determined by flow cytometry analysis. (D) Statistical analysis of MHCII positive cells in each experimental

condition in (C). n=3; mean±SD; * $P<0.05$ compared to the control group without LPS treatment. # $P<0.05$ compared to the control group without LPS treatment. *** $P<0.001$ compared to the control group with LPS treatment. A second experiment provided similar results.

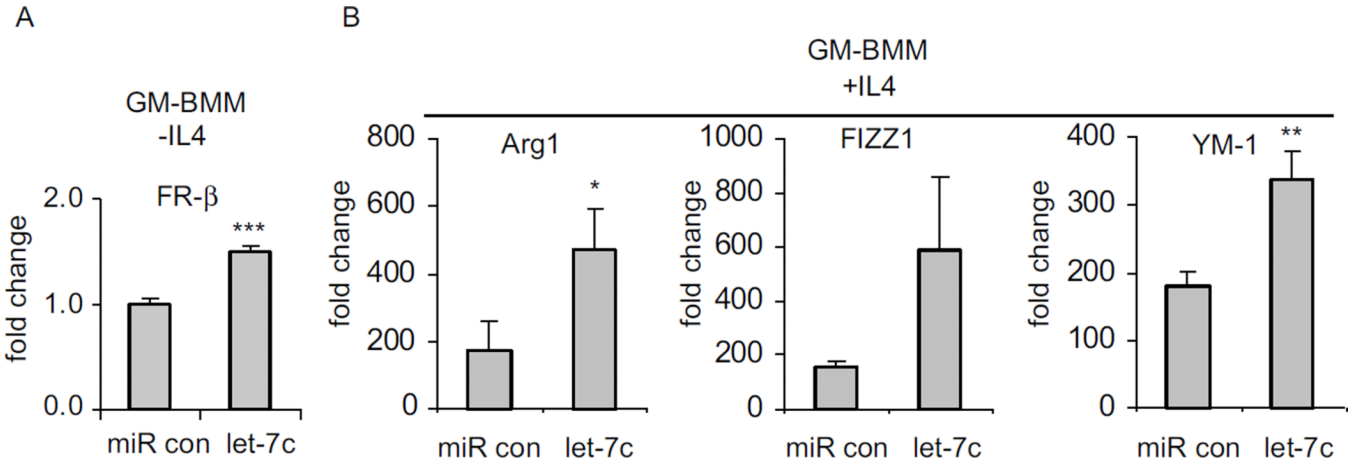

Figure 3. Overexpression of let-7c promotes GM-BMM transition to the M2 phenotype

(A–B) GM-BMM were transfected with 20 nM control mimics or mimics for let-7c. 3 days after transfection, the cells were treated without or with 2 ng/ml IL-4 for 24h. Levels of FR-β (A), and Arg1, FIZZ1 and YM-1 (B) were determined. n=3; mean±SD; * $P<0.05$, ** $P<0.01$, *** $P<0.001$ compared to the control groups. A second experiment provided similar results.

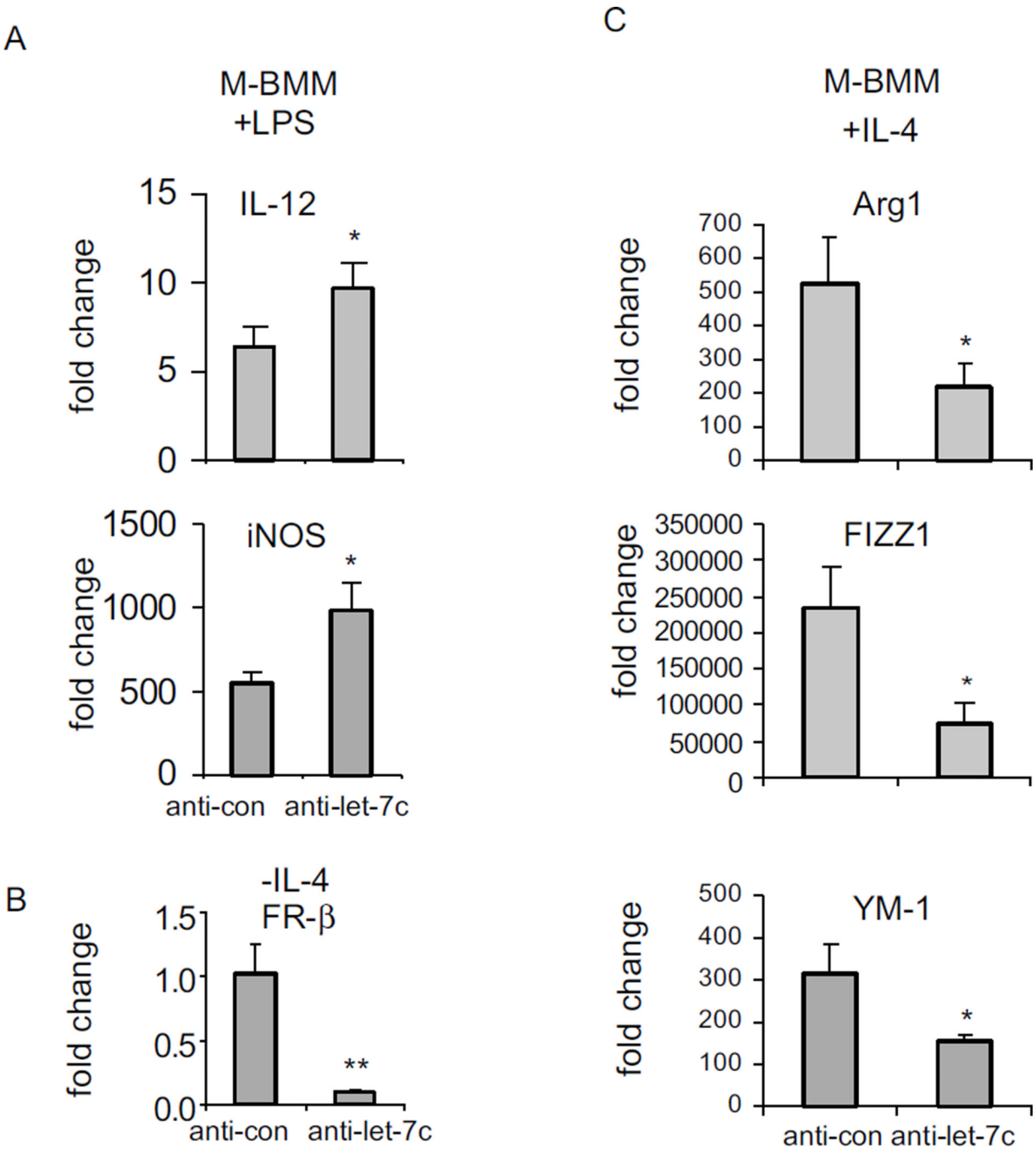

Figure 4. Knockdown of let-7c promotes transition of M-BMM to the M1 phenotype and diminishes the expression of M2 phenotypes in M-BMM

(A) M-BMM were transfected with 20 nM control inhibitors or inhibitors against let-7c. 3 days after transfection, the cells were treated without or with 100 ng/ml LPS for 6h. Levels of IL-12 and iNOS were determined. n=3; mean±SD; * P<0.05 compared to the control groups. (B–C) M-BMM were transfected with 20 nM control inhibitors or inhibitors against let-7c. 3 days after transfection, the cells were treated without or with 2 ng/ml IL-4 for 24h. Levels of FR-β (B), and Arg1, FIZZ1 and YM-1 (C) were determined. n=3; mean±SD; * P<0.05, ** P<0.01 compared to the control groups. A second experiment provided similar results.

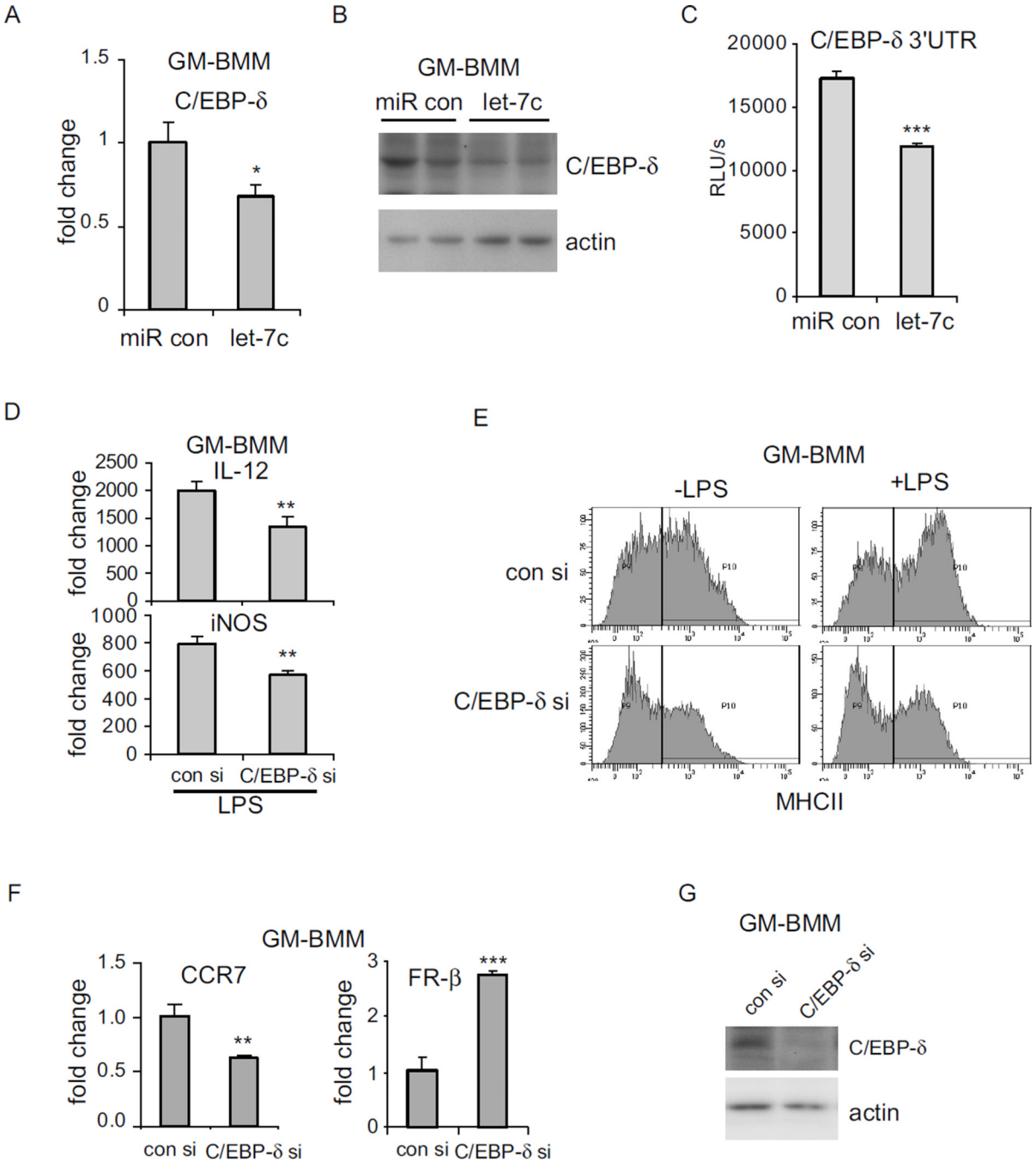

Figure 5. let-7c targets C/EBP-δ

(A) GM-BMM were transfected with 20 nM control mimics or mimics for let-7c. 3 days after transfection, levels of C/EBP-δ were determined by real-time PCR. n=3; mean±SD; * $P<0.05$ compared to the control group. (B) GM-BMM were transfected with 20 nM control mimics or mimics for let-7c. 3 days after transfection, levels of C/EBP-δ and actin were determined by Western blotting. (C) 5 ng pMIR-Reporter-C/EBP-δ were co-transfected with 20 nM control mimics or mimics for let-7c into HEK-293T cells. 24h after transfection, luciferase activity in the cells was determined. n=3; mean±SD; *** $P<0.001$ compared to the control group. (D) GM-BMM were transfected with 20 nM control siRNAs or siRNAs targeting C/EBP-δ. 3 days after transfection, the cells were treated without or with 100 ng/

ml LPS for 6h. Levels of IL-12 and iNOS were determined by real-time PCR. n=3; mean ±SD; ** $P<0.01$ compared to the control group treated with LPS. (E) GM-BMM were transfected with 20 nM control siRNAs or siRNAs targeting C/EBP-δ. 3 days after transfection, the cells were treated without or with 100 ng/ml LPS for 6h. The surface levels of MHCII were determined by flow cytometry analysis. (F) GM-BMM were transfected with 20 nM control siRNAs or siRNAs targeting C/EBP-δ. 3 days after transfection, Levels of CCR7 and FR-β were determined by real-time PCR. n=3; mean±SD; ** $P<0.01$, *** $P<0.001$ compared to the control group. (G) GM-BMM were transfected with 20 nM control siRNAs or siRNAs targeting C/EBP-δ. 3 days after transfection, levels of C/EBP-δ and actin were determined by Western blotting.

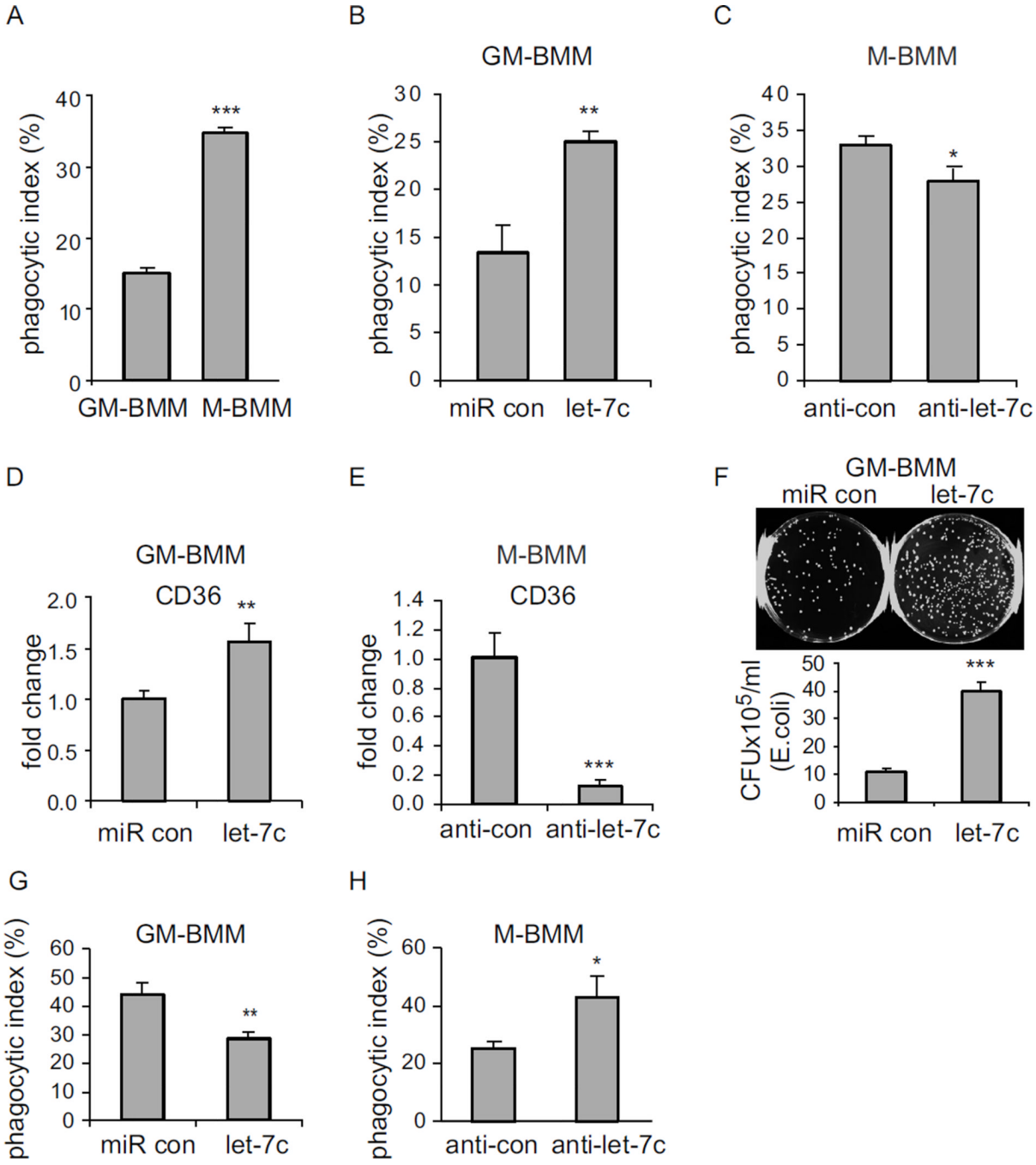

Figure 6. let-7c regulates cellular functions associated with M1 and M2 phenotypes

(A) 0.5×10^6 apoptotic thymocytes were added to GM-BMM and M-BMM macrophages and phagocytosis assays performed. Phagocytic index was calculated as described in "Materials and Methods". n=3; mean±SD; *** $P<0.001$ compared to the control group. (B) GM-BMM were transfected with 20 nM control mimics or mimics for let-7c. Phagocytosis assays were performed as in A. n=3; mean±SD; ** $P<0.01$ compared to the control group. (C) M-BMM were transfected with 20 nM control inhibitors or inhibitors against let-7c. Phagocytosis assays were performed as in A. n=3; mean±SD; * $P<0.05$ compared to the control group. (D–E) GM-BMM or M-BMM were transfected with 20 nM control mimics, mimics for let-7c, control inhibitors or inhibitors against let-7c. Levels of CD36 were determined. **

P<0.01, *** *P*<0.001 compared to the control group. (F) GM-BMM were transfected with 20 nM control mimics or mimics for let-7c. 3 days after transfection, live E. coli were added into the media. 1h after incubation, the supernatants were removed and cultured on LB agar plates at 37 °C for 24h. The bacteria colonies were enumerated and the CFU of E. coli in the supernatants were determined. n=3; mean±SD; *** *P*<0.001 compared to the control group. A second experiment provided similar results. (G–H) GM-BMM or M-BMM were transfected with 20 nM control mimics, mimics for let-7c, control inhibitors, or inhibitors against let-7c. Red fluorescent labeled carboxylate-modified beads opsonized with mouse IgG (5 mg/ml) were then incubated with macrophages for 15 minutes. The cells were washed 3 times and cell suspension prepared. Flow cytometry assays were performed. * *P*<0.05, ** *P*<0.01 compared to the control group.