Title

MiR-146a modulates macrophage polarization by inhibiting Notch1 pathway in
RAW264.7 macrophages

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Abstract

Macrophages are heterogeneous and plastic cells with the ability to undergo dynamic transition between M1 and M2 polarized phenotypes in response to the microenvironment signals. However, the underlying molecular mechanisms of macrophage polarization are still obscure. In the current study, we revealed that miR-146a may play a pivotal role in macrophage polarization. As our results indicated, miR-146a was highly expressed in M2 macrophages rather than M1 macrophages. Over-expression of miR-146a resulted in significantly repressed production of pro-inflammatory cytokines including iNOS and TNF- α in M1 macrophages, whereas increased production of M2 marker genes such as Arg1 and CD206 in M2 macrophages. In contrast, knockdown of miR-146a promoted M1 macrophage polarization but diminished M2 phenotype expression. Mechanistically,

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miR-146a targeted at Notch1 and remarkably downregulated its expression and subsequently polarized macrophages. Of note, miR-146a-mediated macrophage polarization was also modulated by PPAR γ which was found a positive correlation with miR-146a. Taken together, our data suggested that miR-146a served as a molecular regulator in macrophage polarization and a potential therapeutic target to prevent inflammatory diseases.

Keywords: macrophage; polarization; miR-146a; Notch1; PPAR γ

Highlights

1. MiR-146a modulated macrophage polarization;
2. MiR-146a inhibited Notch1 pathway;
3. MiR-146a enhanced the activation of M2 macrophage

Abbreviations

Arg1, arginase 1

iNOS, inducible NO synthase

IL-10, interleukin-10

miRNA, microRNAs

PPAR γ , Peroxisome proliferator-activated receptor γ

TNF- α , Tumor Necrosis Factor alpha,

1. Introduction

Macrophages are heterogeneous and versatile cells that function as immune effectors and regulators, tissue remodelers, or scavengers in both tissue homeostasis and lesions. Available evidence suggested that macrophages could promote the initiation and progression of tissue injury and improve the function of the wound healing and tissue remodeling in various diseases of pathogenesis[1,2]. It is well accepted that activation of macrophages is important to eliminate the invading pathogens or the insulting stimulation. However, the inflammatory signaling must be resolved to prevent the self-destruction while the infectious or injurious agents are

cleared. Thus, the proper activation of macrophages and the transition from an inflammatory to immunosuppressive phenotype are equally important [3,4].

It is generally believed that macrophages show a high degree of plasticity with the ability to undergo dynamic transition between different functional phenotypes in response to the microenvironment signals [5,6]. Upon exposure to microbial products or pro-inflammatory cytokines (IFN- γ , TNF, or TLR ligands), macrophages undergo a phenotype switch referred to as classically activated macrophages or M1 macrophages, which highly produce pro-inflammatory cytokines, e.g. TNF- α , IL-6, IL-1 β and nitric oxide (NO) by expressing inducible NO synthase (iNOS)[7]. In contrast, stimulation of macrophages with IL-4 and IL-13, immune complexes plus TLR ligands, IL-10, TGF- β or glucocorticoids induce the generation of alternatively activated macrophages or M2 macrophages, which produce high levels of M2-associated genes such as mannose receptor (Mrc) 1/2, Chitinase 3-like 3 (Chi3l3, also called Ym1), and arginase 1 (Arg1) [8,9]. M1 macrophages play a crucial role in protecting the host against the infection, like eliminating bacterial, viral and fungal infections, but they also contribute to the pathogenesis of the inflammatory and degenerative diseases [10,11]. On the contrary, M2 macrophages suppress effector T cells and facilitate tumor progression, but they are beneficial for inflammation modulation and tissue remodeling [11,12]. However, the underlying molecular mechanisms of macrophage polarization are still obscure.

Emerging evidence suggested that microRNAs (miRNA) played important roles in macrophage polarization and function[13,14]. These functional miRNAs like miR-155 and miR-21 which are induced by a variety of inflammatory stimuli have been identified associated with polarized macrophages via different pathway or signaling [14,15]. Thus, the importance of miRNAs in determining macrophage polarization has been increasingly appreciated. Previous study by our group showed that miR-146a might function as a novel feedback negative regulator to LPS-induced production of inflammatory cytokines. MiR-146a, located in the second exon of the LOC285628 gene on human chromosomes 5, is involved in human diseases such as the innate immune response, haemopoiesis, inflammatory disease and cancer[16-19]. However,

little is known about the potential role of miR-146a in the regulation of macrophage polarization.

The Notch signaling pathway is an evolutionarily conserved pathway and critically mediates juxtacrine signaling that influences cell-to-cell communication in a variety of tissues and organs throughout the body and is implicated in many genetic and acquired diseases and conditions. Specifically, the Notch pathway is associated with various cell fate controls during cell differentiation, proliferation, and apoptosis [20-22]. Evidence provided suggests Notch signaling plays a critical role in cell fate determination of monocytes and participates in macrophage modulation [23,24]. A recent study provided evidence that over-expression of miR-146a in glioblastomas exerts an anti-tumor effect through downregulation of Notch1, which is a direct target of miR-146a, suggesting that there is an intimate relationship between miR-146a and Notch1 [25]. Therefore the current study engaged to explore whether miR-146a is participate in the regulation of macrophage polarization and the potential underlying mechanisms in the process.

2. Materials and Methods

2.1. Cell Culture and Macrophage Polarization

The RAW264.7 macrophage cell lines (ATCC, USA) were maintained in DMEM (Gibco, USA) supplemented with 10% (v/v) FBS (Gibco, USA), 100 µg/ml streptomycin, 100 U/ml penicillin, and 2 µM L-glutamine (Beyotime, China) and incubated at 37°C and an atmosphere of 5% CO₂. M1 macrophages were generated by treatment with LPS (1000ng/ml, Sigma, GER) and IFN-γ (10ng/ml, Sigma, GER) for 12 h. M2 macrophages were generated by treatment with IL-4 (15ng/ml, Sigma, GER) for 48 h.

2.2. Transient Transfection of miR-146a Mimic and miR-146a Inhibitor

To overexpress and downregulate the expression of miR-146a, macrophages were transfected with miR-146a mimic or inhibitor (Genema, China) by using lipofectamine 2000 (Invitrogen, USA). The miRNA mimic as simulated biological endogenous miRNA, which is synthesized by chemical synthesis method, can

enhance the function of the endogenous miRNA. While the miRNA inhibitor is one kind of cells specific inhibitors of the target miRNA by chemical special modification. Macrophages were plated in DMEM supplemented with 10% FBS at a density of $2-3 \times 10^5$ cells/ml and were transfected with the miR-146a mimic, miR-146a inhibitor, the control miRNA mimic and control miRNA inhibitor. After transfection for 6 h, the culture medium was changed to DMEM from Opti-MEM (Invitrogen, USA). The sequences of oligonucleotides used are as follows: miR-146a mimic, forward, 5'-UGA GAA CUG AAU UCC AUG GGU U-3' and reverse, 5'-CCC AUG GAA UUC AGU UCU CAU U-3'; miR-NC mimic, forward, 5'-UUC UCC GAA CGU GUC ACG UTT-3' and reverse, 5'-ACG UGA CAC GUU CGG AGA ATT-3'; miR-146a inhibitor, 5'-AA CCC AUG GAA UUC AGU UCU CA-3'; miR-NS inhibitor, 5'-UCU ACU CUU UCU AGG AGG UUG UGA-3'.

2.3. RNA Isolation and Quantitative Real-Time PCR

Total RNA was isolated from RAW264.7 macrophage cells using TRIzol reagents (Invitrogen, USA), and the reverse transcription of first-strand cDNA was performed at 37 °C for 15min, 85 °C for 5 s, and 37 °C for 10 min by using a PrimeScript[®]RT reagent kit (Takara, JAP) according to the manufacturer's protocol.

Quantitative real-time-PCR analyses for mRNA of TNF- α , iNOS, Arg1, IL-10, Notch1, Hes1, PPAR γ and GAPDH were performed by using SYBR Green PCT Master Mix (Takara, JAP). The mRNA level of GAPDH was used as an internal control. The sequences of oligonucleotides used are as follows: GAPDH, forward, 5'-GTC TTC ACC ACC ATG GAG -3' and reverse, 5'- CCA AAG TTG TCA TGG ATG ACC-3; TNF- α , forward, 5'-CCT CAC ACT CAG ATC ATC TTC TC-3' and reverse, 5'- AGA TCC ATG CCG TTG GCC AG-3'; Arg1, forward, 5'-TCA TGG AAG TGA ACC CAA CTC TTG-3' and reverse, 5'-TCA GTC CCT GGC TTA TGG TTA CC-3'; IL-10, forward, 5'-GCT CTT ACT GAC TGG CAT GAG-3' and reverse, 5'-CGC AGC TCT AGG AGC ATG TG-3' ; Notch1, forward, 5'-CCA GCA TGG CCA GCT CTG G-3' and reverse, 5'-CAT CCA GAT CTG TGG CCC TGT T-3'; Hes1, forward, 5'- CTC CCG GCA TTC CAA GCT AG-3' and reverse, 5'-AGC GGG TCA CCT CGT TCA TG-3'; PPAR γ , forward, 5'- TCC AGC ATT TCT GCT CCA CA-3'

and reverse, 5'- ACA GAC TCG GCA CTC AAT GG-3'. PCR was performed at 94 °C for 5 min, followed by 30–35 cycles of amplification at 94 °C for 40 s, 51 °C for 40 s, and 72 °C for 1 min by using ABI9700.

2.4. One-Step Quantitative Real-Time PCR

To confirm expression of miR-146a, miR-146a was measured using EzOmics miRNA qPCR Detection Primer Set (Biomics, USA) and EzOmics One-Step qPCR Kit (Biomics, USA). PCR was performed at 42 °C for 30 min, 95 °C for 10 min, followed by 40 cycles of amplification at 95 °C for 20 s, 62 °C for 30 s, and 72 °C for 30 s. The melt curve stage was performed at 95 °C for 30 s, 60 °C for 30 min, and 95 °C for 30 s. Relative expression was calculated by the $\Delta\Delta C_t$ method relative to U6, a normalizing control.

2.5. Western Blotting

Cell lysates were prepared in standard RIPA buffer (Abcam, UK) supplemented with PMSF. Protein lysates were then quantified using the BCA protein assay kit (Boster, China). Equal amounts of protein were separated on 10% SDS-polyacrylamide mini-gels and blotted onto polyvinylidene difluoride membrane (Millipore, USA). After blocking in TBS/Tween (0.075%) containing 3% Marvel, nitrocellulose blots were incubated for 1 h with the appropriate primary antibodies, followed by a secondary Ab conjugated to HRP (Abcam, UK). Proteins were visualized with ECL-chemiluminescent kit (Thermo Scientific, USA). Rabbit anti- α -SMA and PPAR γ were from Bioss (China) and anti- β -actin from Bioworld (USA). Rabbit anti- Arg1, anti-Hes1, anti-iNOS and anti-Notch1 were from Cell Signaling (USA).

2.6. ELISA

The concentrations of TNF- α and IL-10 in the supernatants of each macrophage culture were measured by the quantification enzyme-linked immunosorbent assay (ELISA) kits (Elabscience biotechnology Ltd., Co, China) according to the manufacturer's instruction. Optical density values were detected at 450 nm. Three independent experiments were performed, and each sample was quantified with three replicates.

2.7. Flow Cytometry Analysis

Macrophages were trypsinized and resuspended in ice-cold flow cytometry buffer (2% [v/v] FBS and 2 mM EDTA in PBS). After blocking of the unspecific binding by Fc γ blocker (BD Pharmingen, USA), cells were incubated with APC/CY7 anti-mouse MHC II mAb (Biolegend, USA) for 30 min at 4°C. Cells were washed once and resuspended in flow cytometry buffer with 0.1% DAPI. Flow cytometry events were gated based on forward and side scatter, and F4/80⁺ cells were then selected for the analysis of the M1/M2 marker. Data were acquired on a FACS Calibur flow cytometer (Beckman, USA) and analyzed on FlowJo software (Tree Star, Ashland, OR).

2.8. Statistical Analysis

All of the experiments were repeated at least three times. Unless otherwise indicated, all data were presented as mean $\pm$ SD. The statistical significance of the differences was analyzed by either Student t test for comparison between means or one-way analysis of variance with values of $P < 0.05$ considered to be statistically significant. All of the calculations were performed using the Prism software program for Windows (GraphPad Software).

3. Results

3.1. MiR-146a is highly expressed in M2 macrophages

To determine macrophage polarization and plasticity, we induced M1 and M2 polarization in RAW264.7 respectively. As confirmed by our laboratory, M1 phenotype were polarized by treatment with LPS (1000 ng/ml) and IFN- γ (10 ng/ml) for 12 h, and M2 macrophages were induced with IL-4 (15 ng/ml) for 48 h. As shown in Fig. 1A, iNOS and TNF- α were highly expressed in M1 macrophages while Arg1 and IL-10 were highly expressed in M2 macrophages, confirming the M1 and M2 activating state respectively. One-step quantitative real-time PCR results indicated that miR-146a was highly expressed in M2 macrophages but a lower level in M1 macrophages (Fig. 1B).

3.2. MiR-146a negatively regulates M1 signature gene expression

To characterize the functional significance of miR-146a in macrophage

polarization, RAW264.7 macrophages were transfected with miR-146a mimic, miR-146a inhibitor, or their NC and NS, respectively, and then subjected to LPS and IFN- γ stimulation for 12 h to generate M1 macrophages (Fig.2A). The results showed that in LPS-IFN- γ -induced expressions of TNF- α and iNOS were significantly repressed by miR-146a over-expression, either at protein or mRNA levels(Fig.2B). Conversely, inhibition of miR-146a caused a substantial upregulation of M1-associated genes (Fig. 2C). These results indicated that miR-146a might play a crucial role in M1 macrophages.

To further strengthen these observations, we simultaneously followed the expression of M1 signature gene, MHCII, by flow cytometry analysis. As expected, MHC II was downregulated by miR-146a over-expression, whereas MHC II was upregulated by miR-146a inhibitor (Fig. 2D). Taken together, it was demonstrated that miR-146a constrained the activation of M1 pro-inflammatory macrophages.

3.3. MiR-146a promotes M2 marker gene transcription

To assess the function of miR-146a in the activation of M2 macrophages, RAW264.7 macrophage cells were respectively transfected with miR-146a mimic, miR-146a inhibitor, or their NC and NS before IL-4 treated (Fig.3A). As expected, the expression level of Arg1 was markedly increased by miR-146a over-expression while downregulated by miR-146a knockdown. Interestingly, similar expression changes in IL-10 were found in Fig.3B and 3C. The data also highlighted that miR-146a might also impact on the activation of M2 macrophages and promote the development of M2-polarized macrophages.

3.4. Notch1 is selectively induced by miR-146a to skew the macrophage response

In our bioinformatic analysis of the miR-146a target genes, we paid more attention to Notch1 which has been proved to play an important role in macrophage inflammatory response [26-28]. To further determine whether miR-146a targets Notch1 in macrophages, RAW264.7 macrophages were transfected with miR-146a mimic, miR-146a inhibitor, or their control and then subjected to LPS and IFN- γ stimulation for 12 h to generate M1 macrophages or to IL-4 stimulation for 48h to generate M2 macrophages. As shown in Fig. 4A, over-expression of miR-146a in M1

macrophages diminished both protein and mRNA expression of Notch1 and Hes1, conversely, inhibition of miR-146a caused a moderate increase in the expression of Notch1 and Hes1. Interestingly, over-expression or inhibition of miR-146a in M2 macrophages caused the uniform changes of Notch1 and Hes1 (Fig. 4B). Thereby, it was revealed that miR-146a might repress M1 and drive M2 macrophage polarization by inhibiting Notch1, at least in part.

3.5. PPAR γ is responsible as another target for the miR-146a-mediated M2 macrophage polarization

Peroxisome proliferator-activated receptor γ (PPAR γ), a member of the nuclear receptor superfamily of ligand-dependent transcription factors, is markedly induced in macrophages stimulated with IL-4, and plays an essential role in controlling the alternatively activated macrophage or M2 activation[29]. PPAR γ has been proved to have anti-inflammatory effects by negatively regulating the expression of pro-inflammatory genes induced in response to macrophage differentiation and activation [30-32]. Considering the important role of PPAR γ in M2 macrophages activation, we speculated that miR-146a may also control M2 macrophage polarization by regulating PPAR γ . We examined the expression of PPAR γ when miR-146a was overexpressed or inhibited. The results showed that the over-expression of miR-146a considerably increased PPAR γ expression, whereas interference of miR-146a reduced its production, in both protein and mRNA levels (Fig. 5A, 5B). Thus, the data demonstrated that in addition to inhibiting Notch1, miR-146a may drive the transition of macrophage polarization toward M2 phenotype by activating PPAR γ .

4. Discussion

Increasing evidence showed the underlying molecular mechanisms of macrophage polarization remain largely unclear. In this study, we found that miR-146a served as an anti-inflammatory regulator of phenotypic and functional polarization properties in macrophages by suppressing pro-inflammatory M1 macrophages switch and promoting M2 macrophage polarization, which is achieved, at least partially, through

a Notch1-dependent mechanism. Interestingly the study also demonstrated miR-146a enhanced PPAR γ activation to promote M2 macrophages activation (Fig. 6). The suppositions were based on our findings that enforced over-expression of miR-146a not only blocked M1 macrophages, but also boosted the expression of the anti-inflammatory cytokine IL-10 and M2-featured genes via inhibiting Notch1 pathway. Conversely, blockade of miR-146a in macrophages enhanced M1 macrophages activation whereas diminishing M2 polarization. Our study also revealed a potential relationship between miR-146a and PPAR γ in macrophage polarization while over-expression or knockdown of miR-146a respectively enhanced and inhibited PPAR γ in M2 differentiation. Available evidence in our study revealed a crucial role of miR-146a and the downstream signaling in macrophage polarization and the transition between the pro- and anti-inflammatory phenotypes, which is believed to provide a novel insight into the molecular regulation of macrophage polarization and the related inflammatory disorders.

Macrophages, as an essential component of innate immunity, are capable of differentiating into protean varieties with a range of function [10,33,34]. Macrophages can acquire the transition of distinct functional phenotypes via undergoing different phenotypic polarization in response to various environment cues, such as damaged cells, activated lymphocytes, microbial product, or under different pathophysiologic condition [35]. Accumulating evidence indicated that miRNAs, a kind of non-coding RNAs that cause gene silencing at the posttranscriptional levels, had been proved as important regulators for gene expression in many immune cells including macrophages [15,36]. It was reported that miR-155 promoted the activation of pro-inflammatory macrophages through repressing the negative regulators, including suppressor of cytokine signaling 1 (SOCS1) and SHIP1 [37]. Additionally, miR-155 suppressed the M2 macrophage development by inhibiting the M2-specifying factor, C/EBP β and IL-13R [38,39]. MiR-223 was found to be differentially expressed during macrophage polarization, with upregulation in LPS-treated macrophages, but downregulation in IL-4 treated BMEMs. MiR-223 targeted Pknox1 in regulating macrophage polarization[40]. Moreover, miR-124 repressed the production of

pro-inflammatory cytokines while elevating the expression levels of M2-associated genes TGF- β 1, Arg1, and FIZZ1 by inhibiting the transcription factors C/EBP- α and PU.1 [41,42]. MiR-146a has been demonstrated as an important regulator in biologic processes and plays a key role in the excessive or impaired inflammatory response of macrophages with the deregulation of miRNAs [43]. Our previous study revealed that miR-146a functioned as a negative feedback regulator in LPS-induced RAW264.7 macrophage cells [44]. However, the role of miR-146a in macrophage polarization remains unclear. Thus, in the study, we reveal an unexpected and fundamental role of miR-146a in regulating the macrophage polarization and the immunological homeostasis.

Another point of our finding is the identification of the role of the Notch1 in molecular regulation of macrophage polarization. Notch1 is one of the four Notch proteins which are trans-membrane receptor and highly conserved cell-fate decisions throughout embryonic development and adult life. In current study, it was reported that Notch1 deficiency resulted in decreasing inflammation and regulating inflammatory cytokine expression in macrophages. Inhibition of Notch can markedly suppress Th1 response, which indicated that increased expression of Notch1 can induce M1 macrophages differentiation and pro-inflammatory cytokine expression [27,45]. Study by Meiet al reported that Notch1 acts as a potential target of miR-146a in glioma stem-like cells [25]. Previous study in our laboratory demonstrated that miR-146a influenced inflammatory cytokine IL-6 expression in RAW264.7 macrophages via inhibiting Notch1 [44]. The evidence indicates that Notch1 may play a pivotal role in miR-146a-mediated modulation of macrophage polarization. Clearly, the hypothesis was verified by our results that miR-146a repressed M1 macrophages switch and promoted M2 macrophage polarization via inhibiting Notch1.

With the conjecture that Notch1 may be not the only regulator of miR-146a on macrophage polarization, we suspected that PPAR γ which has been investigated as candidate pathways for integrating inflammatory responses and polarizes macrophages from M1 towards M2 phenotype might be involved in the regulation of miR-146a-mediated M2 macrophage polarization. It was demonstrated that activation

of PPAR γ could skew polarization of human monocytes toward an anti-inflammatory M2 phenotype [46]. Subsequent studies reported that PPAR γ played as an important controller in the maturation of alternatively activated macrophages [29]. Furthermore, PPAR γ expression was strongly correlated with the marker genes expression of macrophages, especially the gene encoding Arg1 [47]. Our data revealed that PPAR γ was involved in the miR-146a-mediated M2 macrophage polarization, supporting a novel regulator mechanism whereby miR-146a and PPAR γ regulated macrophage polarization. However, the detailed mechanism between miR-146a with PPAR γ is unclear, further studies may be required to determine how miR-146a works on PPAR γ to modulate macrophage polarization.

In conclusion, we found that miR-146a suppressed the activation of M1 macrophages, while enhanced the activation of M2 macrophages by inhibiting Notch1. Besides, miR-146a enhanced the activation of M2 macrophage through promoting PPAR γ in addition to inhibiting Notch1. Our studies provided a novel example that macrophage polarization was not modulated by protein regulators only, but also by miRNAs, such as miR-146a. In future research, we will research the potential role that miR-146a plays in inflammation-related diseases. The modulation of miR-146a and the relevant signaling molecules may provide a new therapeutic implication for immune diseases.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Figure legends

Fig.1. MiR-146a is expressed at a low level in M1 macrophages but a high level in M2 macrophages which were generated by treatment with LPS and IFN- γ or IL-4 respectively.

(A) RAW264.7 macrophages were respectively treated with LPS (1000ng/ml), IFN- γ (10ng/ml) for 12 h or IL-4 (15ng/ml) for 48 h to M1-phenotype or M2-phenotype. RNA was isolated and the expression of M1 markers iNOS, TNF- α , and M2 markers Arg1, IL-10 was determined by real-time PCR. n=6; mean $\pm$ SD; *P < 0.05, **P, ##P, \$\$P, &&P < 0.01 compared with untreated normal RAW264.7 macrophages.

(B) Levels of miR-146a in M1 and M2 macrophages were determined by one-step quantitative real-time PCR. SnRNA RNU6 was used as an internal control. n=6; mean $\pm$ SD; **P, ##P < 0.01 compared with untreated normal RAW264.7 macrophages.

Fig.2. MiR-146a suppresses the expression of pro-inflammatory cytokines in M1 phenotype.

(A) RAW264.7 macrophages were transfected with miR-146a mimic, miR-146a inhibitor or their negative control (NC or NS) for 24 h, and then subjected to LPS and IFN- γ stimulation for 12 h to generate M1 macrophages. Levels of miR-146a were

determined by one-step quantitative real-time PCR. n=6; mean $\pm$ SD; **P, ##P<0.01 compared to the control groups.

(B-C) Relative protein and mRNA levels of pro-inflammatory cytokines, iNOS and TNF- α were assayed by Western blotting, ELISA or real-time PCR, respectively. n=6; mean $\pm$ SD; *P, ##P<0.01 compared to the control groups. (D) Expression of MHC II on RAW264.7 macrophage cells was detected by flow cytometry analysis.

Fig.3 MiR-146a promotes M2-featured genes transcription.

(A) RAW264.7 macrophages were transfected with miR-146a mimic, miR-146a inhibitor or their negative control (NC or NS) for 12 h, and then subjected to IL-4 stimulation for 48h to generate M2 macrophages. Levels of miR-146a were determined by one-step quantitative real-time PCR. n=6; mean $\pm$ SD; **P < 0.01 compared to the control groups.

(B-C) Relative protein and mRNA levels of M2-featured genes, Arg1 and IL-10, were assayed by Western blotting, ELISA or real-time PCR, respectively. n=6; mean $\pm$ SD; *P, ##P<0.01 compared to the control groups.

Fig.4 MiR-146a inhibits Notch1 activity to suppress M1 macrophages development and promote M2 macrophage polarization.

(A) RAW264.7 macrophages were transfected with miR-146a mimic, miR-146a inhibitor or their negative control and then treated with LPS and IFN- γ for 12 h to generate M1 macrophages. Relative protein and mRNA levels of Notch1 and Hes1 were determined by Western blotting or real-time PCR. n=6; mean $\pm$ SD; # P < 0.05, **P, ##P<0.01 compared with the control groups.

(B) RAW264.7 macrophages were transfected with miR-146a mimic, miR-146a inhibitor or their negative control (NC or NS) for 12 h, and then subjected to IL-4 stimulation for 48 h to generate M2 macrophages. Relative protein and mRNA levels of Notch1 and Hes1 were determined by Western blotting or real-time PCR. n=6; mean $\pm$ SD; *P, #P<0.05, **P, ##P<0.01 compared with the control groups.

Fig.5 MiR-146a enhances PPAR γ activity to promote M2 macrophage polarization.

(A) RAW264.7 macrophages were transfected with miR-146a mimic or negative control and then treated with IL-4 for 48h to generate M2 macrophages. Relative protein and mRNA levels of PPAR γ were determined by Western blotting or real-time PCR. n=6; mean $\pm$ SD; ** P < 0.01 compared with the control groups. (B) RAW264.7 macrophages were transfected with miR-146a inhibitor or negative control and then treated with IL-4 for 48h to generate M2 macrophages. Relative protein and mRNA levels of PPAR γ were determined by Western blotting or real-time PCR. n=6; mean $\pm$ SD; ** P < 0.01 compared with the control groups.

Fig.6 Proposed model for the regulation of macrophage polarization by miR-146a.

Fig.2**A** **B** **C** **D**

Fig.3**A** **B** **C**

Fig.4

Fig.5**A** **B**

Fig.6