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IL-16 regulates macrophage polarization as a target gene of mir-145-3p

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ABSTRACT

Background: Interleukin 16 is an immunomodulatory chemokine that signals through CD4 + T cells, monocytes, macrophages and dendritic cells. Its expression in immune-related cells enhances the antimicrobial effect and inhibits HIV replication in macrophages. However, the role of IL-16 in macrophage polarization is uncertain. Mir-145 was reported to regulate IL-10 expression by targeting histone deacetylase 11 and promotes alternatively activated macrophage (M2) polarization. Mir-145 was also predicted to target IL-16 mRNA. We aimed to explore the roles of IL-16 and mir-145 in macrophage polarization and antimicrobial functions.

Methods: THP1 monocytes were employed in this study, and their cell activity when incubated with different concentrations of IL-16 was evaluated using the CCK-8 cell counting kit. To obtain polarized macrophages, THP-1 cells were induced by IL-4 and IL-13 following PMA incubation (M2 polarized macrophages) or induced by IFN-gamma and LPS (M1 classical macrophage activation). The influence of IL-16 on macrophage phagocytosis was quantified by the amount of chicken red blood cell phagocytized. IL-16, IL-10 and miR-145 expression in THP1 monocytes and induced macrophages was quantified by quantitative PCR. The miR-145 and IL-16 targeting relationship was verified by the dual luciferase reporter assay. The influence of IL-16 and mir-145 on macrophage polarization was evaluated by M1 and M2 macrophage characterized marker gene expression.

Results: The M0 macrophage subtype was induced by PMA. The M1 and M2 subtypes of macrophage were successfully induced by M1- and M2-specific induction. M1 macrophages express higher levels of IL-16 than M2 macrophages but express lower levels of IL-10 and mir-145 than M2 cells. IL-16 with a concentration up to 150 ng/mL has no influence on THP-1 cell proliferation but improves macrophage phagocytosis ability with the down-expression of IL-10 and up-expression of pro-inflammatory cytokines such as IL-1a and IL-6. Knockdown with its target siRNA is beneficial for macrophage maintenance but reduces phagocytosis ability. Mir-145 specifically targets the IL-16 3'UTR verified by the dual luciferase reporter assay. Mir-145 downregulates IL-16 expression and upregulates IL-10 expression, thereby promoting M2 macrophage polarization.

Conclusion: IL-16 modulates macrophage polarization through regulating IL-10, IL-1a and IL-6 expression. Mir-145 is involved in M2 macrophage polarization by targeting IL-16 and enhancing IL-10 expression.

1. Introduction

The inflammation response to pathogen infection or native damage factors is subtly regulated by the innate immune system. As part of the vertebrate innate immune system, macrophages, which are derived from bone marrow monocytes, function as crucial control switches of the immune system, securing the balance between pro- and anti-inflammatory reactions. For this purpose and depending on the activating stimuli, these cells can develop into different subsets: pro-inflammatory classically activated (M1) or anti-inflammatory alternatively activated macrophages (M2). This terminology corresponds to T helper type 1 (Th1) (C57BL/6 mice) and T helper type 2 (Th2) (Balb/c mice) cells. Th1 mice with T cells producing mostly IFN-gamma demonstrated macrophage activation in which nitric oxide (NO) was generated from arginine versus ornithine production from Th2 mice with T cells producing IL-4 and tumor growth factor beta 1(TGF-β1) (Mills et al., 2000). Generally,M1 macrophages are characterized by the production of high levels of pro-inflammatory cytokines, the ability to mediate

Abbreviations: IL-16, interleukin 16; IL-1a, interleukin 1 alpha; IL-6, interleukin 6; LPS, lipopolysaccharide; PMA, phorbol 12-myristate 13-acetate; IFN-γ, interferon gamma; M0, non-polarized macrophage; M1, classically activated macrophage; M2, alternatively activated macrophage

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resistance to pathogens, strong microbicidal properties, high production of reactive nitrogen and oxygen intermediates, and promotion of Th1 responses. By contrast, M2 macrophages are characterized by their involvement in parasite control, tissue remodeling, immune regulation, tumor promotion and efficient phagocytic activity. Classical activation of macrophages occurs following injury or infection. Macrophages are classically activated in vitro using the bacterial cell wall components LPS and IFN-γ or TNF-α. Alternatively, activated macrophages polarized by IL-4 and IL-13 are characterized by the limited production of pro-inflammatory cytokines, but they secrete anti-inflammatory cytokines like IL-10, CCL18 and CCL22 (Genin et al., 2015). There are many literatures demonstrating different activation pathways outside the traditional M1/M2 phenotype. To standardize the terms to use, Murray PJ et al suggested an original identical nomenclature which indicates the culture systems and activators used for the differentiation of macrophage (Murray et al., 2014). For example, the M(IL-4), M(IL-10) are considered to be subtypes of M2 macrophage; M(IFN-γ), M(LPS),M (LPS + IFN-γ) are subtypes of M1 macrophage. The M1 and M2 macrophages mentioned in our work were M(LPS + IFN-γ) and M(IL-4) macrophages respectively based on the nomenclature.

IL-16 is a multifunctional cytokine that was first characterized with chemoattractant activity for human T cells in 1982 (Center and Cruikshank, 1982). Cells of origin for IL-16 include immune cells (such as T cells, eosinophils, neutrophils (Roth et al., 2015), monocytes (Elssner et al., 2004) and dendritic cells) and nonimmune cells (such as fibroblasts, epithelial cells, and neurons) (Cruikshank et al., 2000). The CD4+ monocytoid cell line THP1 has also been shown to synthesize as well as respond to IL-16 (Cruikshank et al., 2000; Blaschke et al., 1999). Caspase-3 cleavage of pro-IL-16 protein produces mature IL-16 and is necessary for IL-16 secretion (Zhang et al., 1998). IL-16 induces the chemotaxis of CD4+ cells such as lymphocytes, eosinophils, and dendritic cells by ligating CD4 directly (Cruikshank et al., 2000) and prevents HIV-1 proliferation in infected CD4+ lymphocytes by transcription inhibition (Baier et al., 1995). For immune modulation, IL-16 was shown to affect T-cell activation and proliferation. IL-16 expression can be triggered by S. aureus infection and is responsible for MRSA pneumonia (Ahn et al., 2014). IL-16 has multiple functions in the immune system, but its role in Th1 and Th2 immune-related processes remains unclear.

Many miRNAs are involved in inflammation by either diminishing or potentiating the inflammatory response through targeting important regulators in signaling networks. For macrophage polarization, it was reported that the overexpression of miR-124 diminished M1 polarization and enhanced M2 polarization in bone marrow-derived macrophage cells (BMDMs) (Ponomarev et al., 2011). MiR-223 was found to be differentially expressed during macrophage polarization, with upregulation in LPS-treated macrophages but downregulation in IL-4-treated BMDMs. The importance of macrophage miR-223 was also confirmed by increased adipose tissue inflammation and insulin resistance in mice with transplantation of bone marrow from miR-223-deficient mice. Pknox1 was identified in these studies as a genuine target of miR-223 (Zhuang et al., 2012). MiR-145 is another reported miRNA that promotes IL-10 expression in TLR4-triggered macrophages through directly targeting the epigenetic IL-10 gene silencer histone deacetylase 11 (Li et al., 2013; Shinohara et al., 2017). It also reported to inhibited Arf6, IL-1β, TNF-α and IL-6 expression as well as phosphorylation of p65 in NF-κB pathway in THP-1 cells through targeting Arf6 (Li et al., 2018). Mir-145 immune suppressing function was also demonstrated by researches from Shi J et al. This work indicated miR-145 directly targeted Akt3, inhibiting NF-κB and mTOR downstream genes (Shi et al., 2018). As a predicted targeting gene of miR-145, we speculate that IL-16 may also be involved in macrophage polarization. This work was aimed to define the role of IL-16 in macrophage polarization.

2. Materials and methods

2.1. Cell culture and treatment

Human monocytic leukemia THP-1 cells were purchased from the Kunming Institution of Zoology. (Yunnan, China). The cells were cultured in RPMI-1640 (Gibco, Thermo Fisher Scientific, USA) containing 10% heat-inactivated FBS (Cellmax, Lanzhou, China) in a 5% CO_2 atmosphere at 37 °C. THP-1 cells were treated with 150 ng/mL of phorbol 12-myristate 13-acetate (PMA; P8139, Sigma, USA) followed by 24 h of incubation in RPMI medium to obtain macrophage-like M0 cells. The macrophages were polarized to M1 macrophages by incubating with 20 ng/mL of IFN-γ (#285-IF; R&D system,USA) and 10 pg/ml of LPS (#8630; Sigma, USA) for 18 h. Macrophage M2 polarization was performed by incubating with 20 ng/ml of interleukin 4 (#204-IL; R&D Systems,UAS) and 20 ng/ml of interleukin 13 (#213-ILB; R&D Systems) for 72 h. siRNAs and mimics were transfected with jetPEI®-Macrophage transfection reagents (103-05 N; Polyplus transfection, BD) according to the manufacturer's protocol.

2.2. Real-time RT-PCR

Total RNA was isolated from cells using the TRNzol Universal RNA extract kit (Tiangen, Beijing, China) according to the manufacturer's protocol. Next, 1 μg of total RNA was reverse transcribed into cDNA using the FastKing RT Kit (Tiangen, Beijing, China) for mRNA detection. To detect miRNA expression, 2 μg of total RNA was reverse transcribed into cDNA using the miRcute Plus miRNA First-Strand cDNA Synthesis Kit (Tiangen, Beijing, China), and a poly A tag was added to the 3′ end of miRNA during cDNA synthesis using the kit. The SuperReal PreMix Plus (SYBR Green) and miRcute Plus miRNA qPCR Detection Kit (Tiangen, Beijing, China), which includes a miRNA reverse primer (Tiangen, Beijing, China), were employed for real-time PCR. PCR was performed using the Mx3000 P Real-Time PCR System (Agilent, USA). The following primer sequences were used: TNF-α sense, 5′-GCTGCACTTTGGAGTGATCG-3′; TNF-α antisense, 5′-TCACT CGGGGTTCGAGAAGA-3′; IL-16 sense, 5′-GGAATCGTGCTTCAGAC CCA-3′; IL-16 antisense, 5′-CTCTGGGCTCCTTTGTCAGG-3′; IL-6 sense, 5′-AGTGAGGAACAAGCCAGAGC-3′; IL-6 antisense, 5′-AGCTGCGCAGA ATGAGATGA-3′; IL-10 sense, 5′-CGAGATGCCTTCAGCAGAG-3′ ; IL-10 antisense, 5′-CGCCTTGATGTCTGGGTCTT-3′; CD163 sense, 5′-AGTCT GCTCAAGATACACAGAAA-3 ; CD163 antisense, 5′-GGGGTAGAAAGG GCAACTCC-3′; CD206 sense, 5′−CCATCGAGGAAGAGGTTCGG-3′; CD206 antisense, 5′-GGTGGGTTACTCCTTCTGCC-3′; GAPDH sense, 5′-CAACCCATGGCAAATTCCATGGCA-3′; and GAPDH antisense, 5′-TCTAGACGGCAGGTCAGGTCCACC-3′ ; mir-145 sense, GGATTCCT GGAAATACTGTTCT ; U6 sense, 5′-CTCGCTTCGGCAGCACA-3′. GAPDH was used as housekeeping gene.

2.3. Enzyme-linked immunosorbent assay

IL-6,IL-10,TNF-α,IL-12 and IL-16 secretion was detected by Enzyme-linked Immunosorbent Assay (Elisa). For cell lyse,100 u L RIPA lysis buffer (0.5 M Tris−HCl, pH 7.4, 1.5 M NaCl, 2.5% deoxycholic acid, 10% NP-40, 10 mM EDTA was added to per 1×10^6 cells. The lysate was assayed by IL-6, IL-10, TNF-α, IL-12 and IL-16 Elisa kits purchased from Elabscience, Wuhan, China according to manufacturer's protocol. Value OD 450 was detected with an Synergy H1 micropate reader (BioTek Instruments,Inc). The concentration of certain cytokines in cell lysate was quantified by standard curve. The expression fold change of the cytokines was concluded by compared to control group.

2.4. Western blot

Cells were lysed with RIPA lysis buffer. The extracted proteins were separated by 10% SDS-PAGE gel electrophoresis. The proteins then

were transfered to PVDF membrane for immunoblotting. Block the membrane for 1 h at room temperature using blocking buffer(5% BSA in TBST) and then incubate membrane with appropriate dilutions of primary antibody in blocking buffer overnight at 4 °C. Wash the membrane in TBST. Incubate the membrane with the recommended dilution of HRP conjugated secondary antibody in blocking buffer at room temperature for 1 h. Wash the membrane in TBST. For signal development, a Pro-light HRP Chemiluminescent Kit(Tiangen, Beijing, China) was used. The plots were analyzed by ImageJ to quantitate the grey value and adjusted to fold change (FC) compared to control group. Primary antibodies used in this research are IL-16 antibody (ab207181, Abcam, USA) and β-actin antibody(E-AB-20031,Elabscience,China).

2.5. CCK8 assay

The CCK8 assay was performed using the Enhanced Cell Counting Kit-8 (Beyotime, China) following the manufacturer's protocol. Briefly, 2000 THP-1 cells were plated onto 96-well plates and were allowed to adhere for 16 h. Next, the cell medium was changed to fresh medium containing different concentrations of IL-16. Cell activity was detected at 0, 6, 24, 48, and 72 h of incubation. The OD 450 absorbance was measured to evaluate cell activity.

2.6. Phagocytosis assay

The phagocytosis assay of M0 cells incubated with IL-16 was performed using the red blood phagocytosis method as described by (Hamczyk et al., 2015a). Specifically, control M0 macrophage cells and IL-16-treated M0 macrophages, which were incubated with medium containing 150 ng/mL of IL-16 for 24 h, were seeded onto plates and incubated with starvation medium (RPMI 1640 medium containing 0.1% heat-inactivated FBS, 2 mM L-glutamine, 100 U/mL of penicillin, and 100 μg/mL of streptomycin) for 2 h at 37 °C in a 5% CO_2 atmosphere. Chicken red blood cells (Gaining Biological, China) were opsonized with rabbit anti-chicken IgG/Y (303-005-003, Jackson, USA), and then the macrophages were incubated with medium containing RBC/IgG complexes for 2 h at 37 °C in a 5% CO_2 atmosphere. The macrophages were then fixed by incubating with 4% formaldehyde for 10–15 min at room temperature. Giemsa solution (G1010; Solarbio, China) was used for erythrocyte staining, and 100 cells were randomly counted for each group. The percentage of phagocytic cells and average blood cell uptake in phagocytic cells were quantified to evaluate phagocytosis activity.

2.7. Apoptosis assay

For the apoptosis assay, an Annexin V-FITC Apoptosis Detection Kit (Beyotime, China) was employed. Cells were dispersed with 0.25% EDTA-trypsin (Solarbio, China), collected and washed with PBS. Next, 10^6 cells were resuspended in 200 μl of binding buffer. Thereafter, 5 μl of Annexin V-FITC and 5 μl of PI solution were added, and the cells and reagents were mixed by pipetting gently, followed by incubation for 20 min with resuspension 2–3 times with a pipette during incubation. The cells were then analyzed by flow cytometry using a FACS calibur system (BD Biosciences).

2.8. Analysis of CD11b, CD 163 and CD206 expression by flow cytometry

CD11b+, CD163+, or CD206+ cells were quantified by flow cytometry antibody labeling. Briefly, cells were dispersed with 0.25% EDTA-trypsin and were washed with PBS. For each group, 10^6 cells were resuspended in 500 μl of PBS and were labeled with 5 μl of FITC-conjugated CD 11b antibody (ab24874; Abcam) or 5ul FITC-conjugated CD163 antibody (orb13881, biorbyt, UK) or 5 μl of Alexa Fluor® 488-conjugated CD206 antibody (ab195191; Abcam). The cells were incubated at room temperature for 30 min with resuspension 2–3 times with pipette during incubation. The cells were then analyzed by flow cytometry using a FACS Calibur system (BD Biosciences).

2.9. Dual luciferase reporter assay

The 3′UTR of IL-16 was synthesized starting with the sequences " TGGTCTCTAGGCTGG" and "GCAGCCCTACTTCT" and was cloned into the pmiRGLO vector. HEK293 cells were cultured on a 12-well plate and were transfected with 100 ng of a reporter plasmid, 200 nM of miR-145-3p mimic or both in each well. Firefly and Renilla activities were determined 24 h after transfection using the Dual Luciferase Reporter Gene Assay Kit (Beyotime, China).

2.10. Data analysis

Each of these experiments repeated three times. For Real-time pcr analysis, fold change expression of gene interest is quantified by $2^{(-\triangle\triangle Ct)}$,in which $\triangle CT = (CT_{\text{gene of interest}} - CT_{\text{gene of housekeeping}})$,$\triangle CT = (\triangle Ct_{\text{induced}} - \triangle CT_{\text{normal}})$. All data were analyzed by the statistical software SPSS 19.0, according to the following procedure: the Levene's test was performed first, and ANOVA was performed when $P > 0.05$, Kruskal-wallis test was performed when $P \leq 0.05$. In the case that the ANOVA results were significant ($P \leq 0.05$), the Dunnett test was further used for the multiple comparison test. When there was no significant difference in ANOVA results ($P > 0.05$), the statistics were ended. When the kruskal-wallis test results were significant ($P < 0.05$), then the ANOVA multiple comparison test of kruskal-wallis was further used. When the results of kruskal-wallis test were not significant ($P > 0.05$), the statistical conclusion was concluded.

3. Results

3.1. M0, M1 and M2 macrophages differentiate from THP1 monocytes

Human THP-1 monocytes were used in this study. To obtain macrophages, THP-1 cells were incubated in the presence of phorbol 12-myristate 13-acetate (PMA). A 24-h incubation in the presence of 150 nM PMA followed by 24-h incubation in control medium was finally used as the differentiation protocol based on the Marie Genin method (Genin et al., 2015). When the cells became adherent, they were considered to be M0 macrophage cells. The expression of the recognized macrophage marker CD11b was analyzed by fluorescence activated cell sorting (FACS). CD11b positive(CD11b+) cells are significantly increased after PMA incubation (Fig. 1, **C**). For M1 macrophage induction, 10 pg/mL of LPS and 20 ng/mL of IFN-γ were added to the medium without PMA and then were incubated 18 h. For M2 macrophage induction, 20 ng/ml of IL-4 and 20 ng/ml of IL-13 were used (Fig. 1, **A**). PMA induced primary M0 macrophages, and two polarized macrophages adhered to the plates (Fig. 1, **B**). Many reports have indicated that TNF-α and IL-6 showed increased expression in M1 macrophages, and IL-10 and CD 163 expression was induced by M2 polarization (Genin et al., 2015; Roszer, 2015; Duluc et al., 2007); thus, we detected the expression level of these genes in different macrophage groups. The Q-PCR results showed that TNF-α and IL-6 were successfully induced in M1 cells compared with both THP-1 monocytes and M0 macrophages, but the IL-10 and CD 163 levels were not altered by LPS and IFN-γ in both M0 and M1 cells(Fig. 1, **D**). The expression levels of IL-10 and CD 163 were specifically induced in M2-polarized cells (Fig. 1, **D & E**). Enzyme-linked Immunosorbent Assay also showed IL-6 was up regulated after LPS and IFN-γ induction, and IL-10 was raised after IL-4 and IL-13 incubation (Fig. 1, **F**). These results are consistent with previous reports for M1 and M2 macrophage polarization.

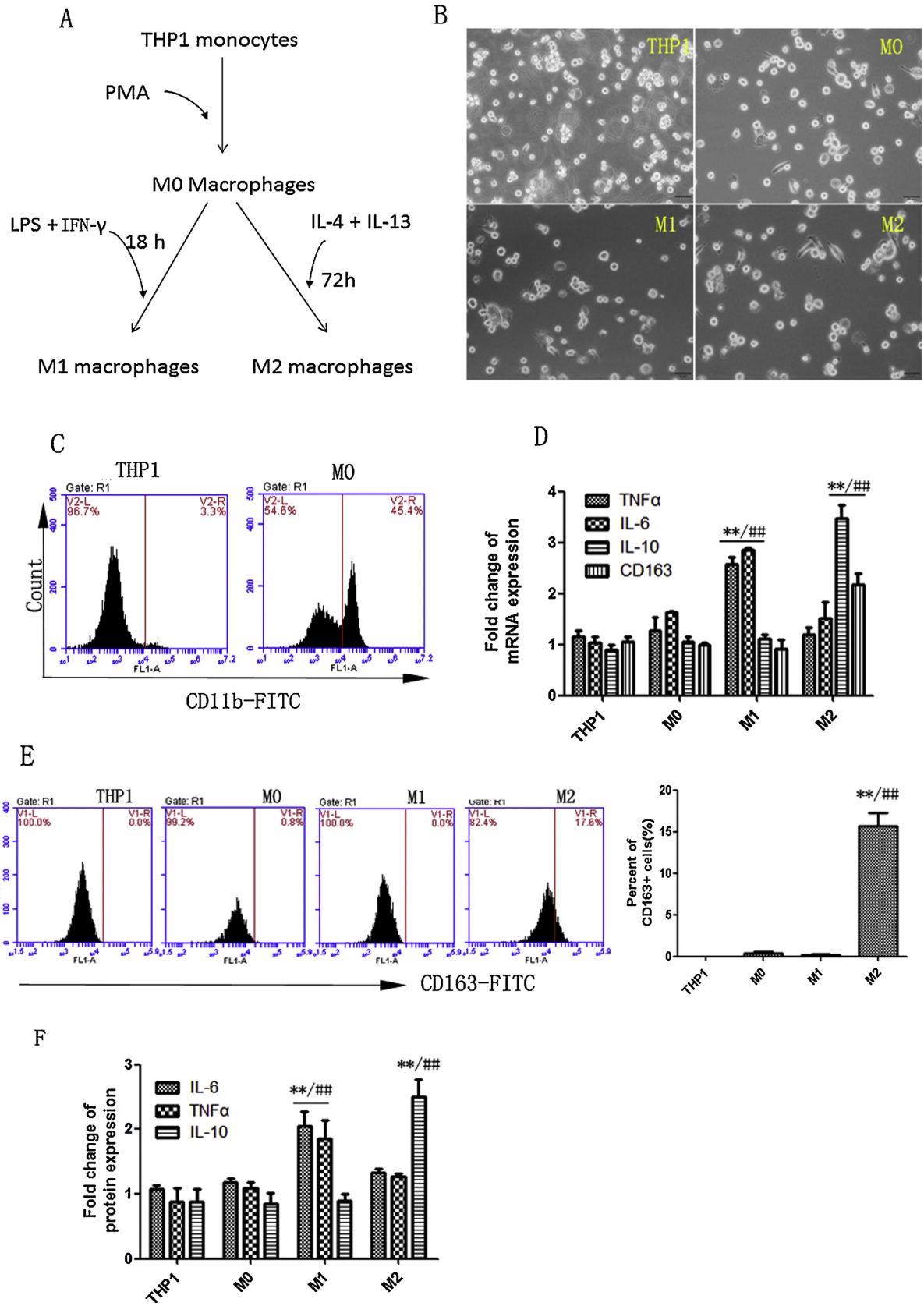

Fig. 1. M0, M1 and M2 macrophages differentiate from THP1 monocytes. (A) Macrophage differentiation work flow. THP-1 cells plated on culture plates were incubated with 160 nM PMA in RPMI 1640 medium containing 5% FBS for 48 h. For M1 macrophage induction, 10 pg/ml of LPS and 20 ng/ml of IFN-γ were added to the medium without PMA. For M2 macrophage induction, 20 ng/ml of IL-4 and 20 ng/ml of IL-13 were added to the medium. (B) Suspended THP1 cells became adherent with an enlarged cell scale after induction. (C) CD11b expression was induced in M0 macrophage cells. (D) Induced M1 and M2 macrophages expressed different macrophage markers. (E) CD163 expression in THP1, M0, M1 and M2 was assayed by FACS. (F) IL-6, TNF-α and IL-10 expressions were detected by Elisa. **P ≤ 0.01 compared with THP1 cells; ##P ≤ 0.01 compared with M0 macrophages. n = 3.

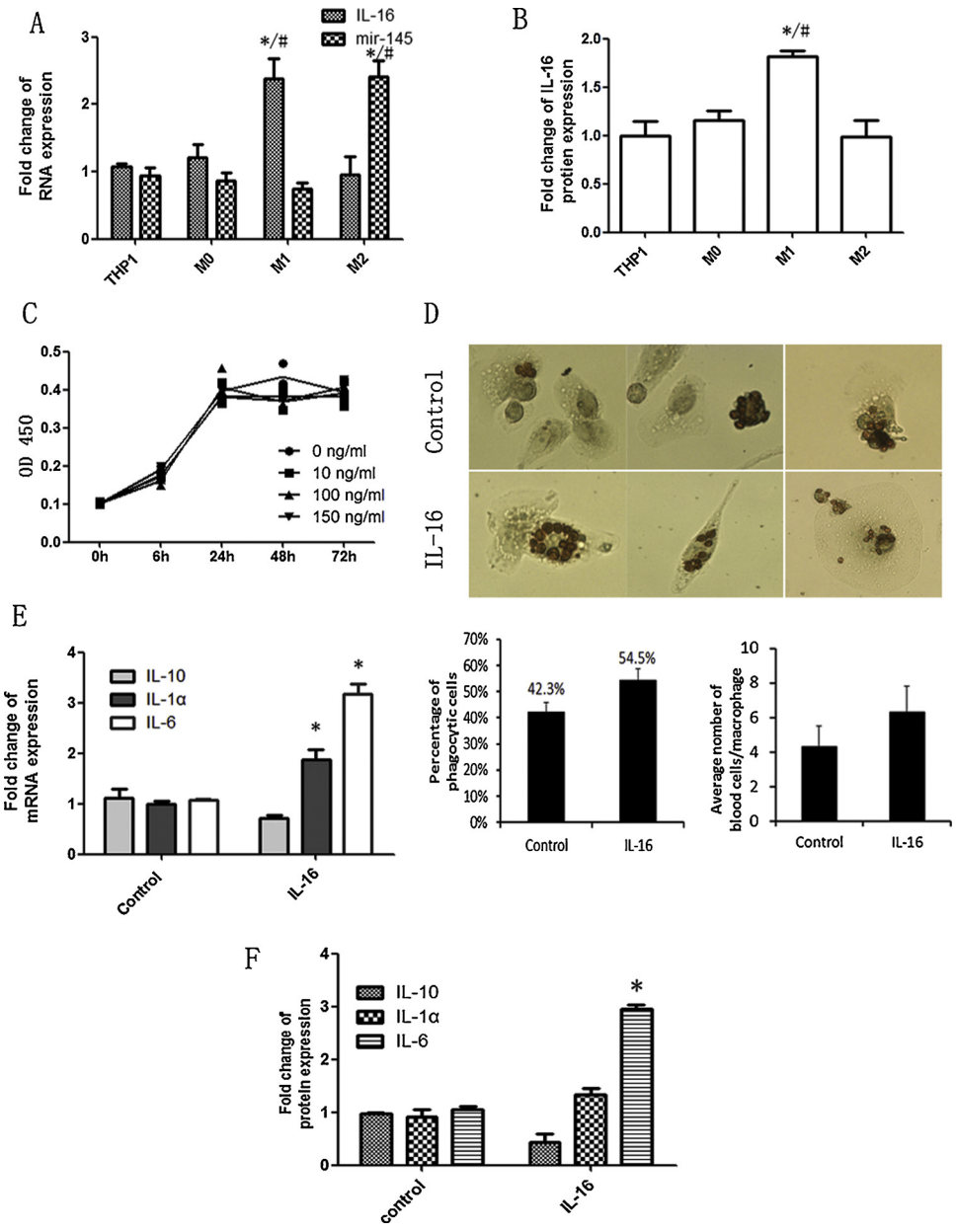

Fig. 2. (A) IL-16 and mir-145 expression in THP-1 cells and differentiated macrophages. (B) IL-16 secretion is up regulated in M1 cells. (C) CCK-8 assay evaluating the influence of three increasing concentrations of IL-16 on THP-1 cell activity. (D) Phagocytosis of macrophages evaluated by chicken red blood cell uptake. One hundred cells were randomly selected in each group to count the number of phagocytic cells among them. The average blood cell uptake in phagocytic cells was counted in each group. (E) IL-10, IL-1a and IL-6 expression was quantified by Q-]PCR in control M0 cells and IL-16-incubated M0 cells. (F) IL-10, IL-1a and IL-6 expression was quantified by Elisa in control M0 cells and IL-16-incubated M0 cells. For (A) and (B),* $P \leq 0.05$ compared with THP1 cells; # $P \leq 0.05$ compared with M0 macrophages. For (E) and (F), * $P \leq 0.05$ compared with control M0 cells. n = 3.

3.2. IL-16 and mir-145 are differently expressed in THP1 monocytes and two macrophage subtypes

Previous reports have indicated that miR-145 regulates the polarization of tumor-associated macrophages and peritoneal exudate macrophages. Another report revealed that IL-16 is constitutively present in peripheral blood monocytes and is spontaneously released during apoptosis (Elssner et al., 2004). We speculated whether miR-145 and IL-16 expression varied during THP-1 monocyte differentiation. Our real-time PCR quantification results showed that IL-16 is significantly upregulated in M1 polarization cells (Fig. 2**, A**). The Elisa results further confirm this upregulation (Fig. 2, **B**). Although miR-145 showed slightly decreased expression in M1 cells, it is induced by M2 polarization with IL-4 and IL-13 incubation (Fig. 2**,A**). Thus, IL-16 and miR-145 might be involved in macrophage polarization.

3.3. IL-16 promotes phagocytosis of macrophages with upregulation of IL-1α and IL-6 expression

To investigate IL-16 function in macrophages, we incubated IL-16 recombinant protein with M0 macrophages. Because macrophages have negligible proliferation activity, before conducting experiments on M0 cells, we used different concentrations of IL-16 to verify its influence on cell activity with THP-1 cells. The CCK8 results showed that IL-16 at a concentration up to 150 ng/mL has no influence on the THP-1 cell proliferation activity (Fig. 2**,C**). Thus, we used 150 ng/mL of IL-16 in subsequent experiments. The phagocytosis assay of M0 cells incubated with IL-16 was performed using the red blood phagocytosis method as described by Hamczyk et al (Hamczyk et al., 2015b). The percentage of phagocytized red blood cells was 54.5% ± 4.2% in IL-16-incubated M0 cells, a value higher than that in control M0 cells (42.3% ± 3.4%). The average number of phagocytized blood cells in macrophages was even greater in IL-16-incubated cells than in control M0 cells (Fig. 2**, D**), though did not reach to a significant difference. This finding indicates that IL-16 may play a pro-phagocytosis role in macrophages. Interestingly, we confirmed that IL-1α and IL-6 were statistically significantly upregulated in IL-16-incubated M0 cells, while IL-10 was slightly downregulated (with no statistical significance) (Fig. 2**, E & F**). This finding suggested that IL-16 may have a pro-M1-polarization function in M0 cells.

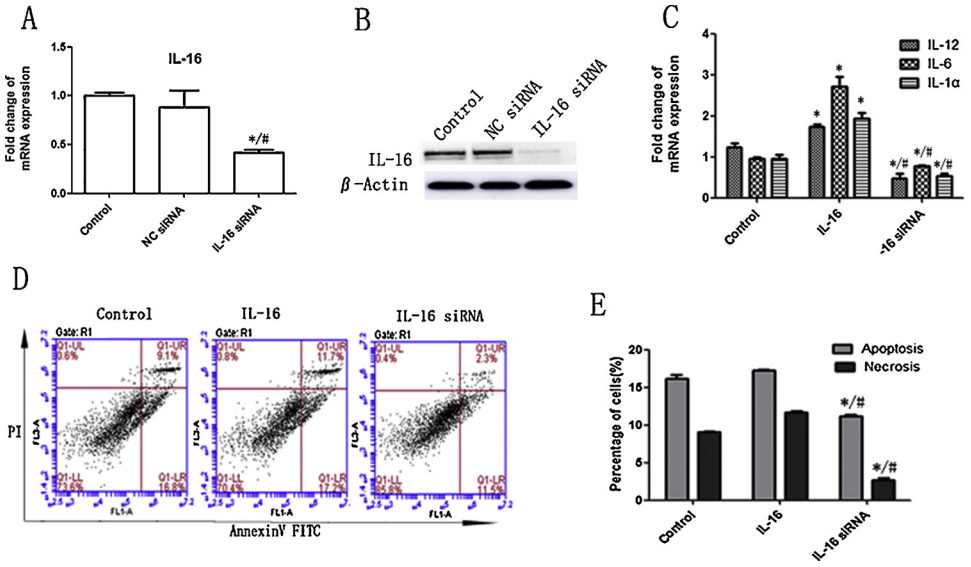

Fig. 3. IL-16 regulates M0 macrophage maintenance and polarization. (A) IL-16 expression was quantified by Q-PCR after 24 h of transfection with siRNAs. (B) IL-16 protein expression was quantified by western blot. (C) Quantitative PCR verification of IL-12, IL-6 and IL-1α expression in M0 macrophage cells incubated with 150 nM of IL-16 protein and cells transfected with IL-16 siRNA. (D) FACS assay of the apoptosis of IL-16-incubated M0 cells and IL-16-knockdown M0 cells. (E) Percentages of apoptosis and necrosis of IL-16-incubated cells and IL-16-knockdown cells were presented as histograms. *P ≤ 0.05 compared with control M0 cells, # P ≤ 0.05 compared with NC siRNA (A) or IL-16-incubated cells (C,E). n = 3.

3.4. IL-16 regulates M1 macrophage maintenance and polarization

Because IL-16 induced M0 macrophage expressing M1 characteristic genes IL-10 and IL-6, we speculated whether IL-16 knockdown could suppress the expression of these genes. We employed an IL-16 siRNA pool (c-39647; Santa Cruz) to knock down IL-16 expression in M0 macrophage cells (Fig. 3 **A, B**). It was show that IL-6 and IL-1α expressions were blocked by IL-16 knockdown (Fig. 3C). The expression of another M1 marker gene, IL-12, was also induced following IL-16 incubation but was suppressed by IL-16 siRNA (Fig. 3B). The apoptosis assay with Annexin V-FITC/PI staining showed that incubation with 150 ng/mL of IL-16 had no influence on M0 cell apoptosis, but IL-16 knockdown suppressed the apoptosis and necrosis of M0 macrophages (Fig. 3**D, E**).

3.5. Mir-145 targets IL-16 and regulates IL-10 expression in M0 macrophages

MiR-145 was reported to promote IL-10 expression in TLR4-triggered macrophages through directly targeting the epigenetic IL-10 gene silencer histone deacetylase 11(Li et al., 2013; Shinohara et al., 2017). It was also predicted to target IL-16 mRNA on the 3′UTR. The online software Targetscan (http://www.targetscan.org) predicted two target sequences on the 3′UTR of IL-16 (Fig. 4**A**). Thus, we cloned sequences containing both targets into the luciferase reporter plasmid pmiRGLO vector. Luciferase reporter plasmids containing the IL-16 3′-UTR were cotransfected into HEK293 T cells with miR-145-3p or control miRNA. Twenty-four hours after transfection, the luciferase activity was measured. It was shown that miR-145-3p significantly reduced the luciferase activity compared with cells transfected with the control plasmid and control miRNA (Fig. 4B). We further verified the regulation of mir-145-3p on IL-16 and IL-10 expression in M0 cells. It was shown that cells transfected with mir-145-3p mimic showed suppressed expression of IL-16 but increased expression of IL-10 (Fig. 4C).

3.6. Downregulation of IL-16 with mir-145 and IL-16 siRNA promotes M2 macrophage polarization

Our previous work indicated that IL-16 recombinant protein promotes M1 macrophage polarization by enhancing IL-12, IL-6 and IL-1αexpression. Knocking down IL-16 with siRNA suppressed the expression of genes, and miR-145 mimic transfection enhanced the expression of IL-10, an M2 macrophage marker gene. We next asked whether IL-16 suppression with miR-145 or IL-16 siRNA could be beneficial for M2 macrophage polarization. By transfecting M0 cells with IL-16 siRNA and miR-145 mimic, we downregulated IL-16 expression in M0 cells. These M0 cells were then subjected to the M2 polarization process described in Fig. 1**A**. Control M0 cells and M2 polarized cells were evaluated by CD206 staining and FACS assay. It was shown that IL-16 siRNA and miR-145-3p both enhanced CD206 expression during M2 polarization (Fig. 5A**, B**). Q-PCR quantification results showed that the expression levels of M2 macrophage marker genes CD206 and IL-10 were enhanced in the IL-16 siRNA- and miR-145-3p-transfected groups (Fig. 5**C, E**). The expression of another gene, CD163, showed no significant difference among between M2-polarized cells and mir-145 or IL-16 siRNA-transfected M2-polarized cells (Fig. 5D).

4. Discussion

Macrophages are among the most versatile cells of the body. Heterogeneity arises because macrophages differentiate from monocyte precursors, and the types are determined by genetic modification as well as by specific tissue-related and immune-related stimuli (Mills et al., 2000; Gordon and Taylor, 2005; Van Ginderachter et al., 2008; Other Functions OGA and Mosser, 2008). A classical activation profile occurs in a type I cytokine environment (IFN-γ, TNF-α) or upon recognition of pathogen-associated molecular patterns (e.g., LPS, lipoproteins, dsRNA, and lipoteichoic acid) and endogenous “danger” signals (e.g., heat shock proteins, extracellular matrix components, and HMGB1) (Other Functions OGA and Mosser, 2008). As such, it plays an important role in protecting against intracellular pathogens and, under certain conditions, cancer cells. Classically activated or M1 macrophages typically produce high levels of IL-12, IL-6 and TNF-α (Gordon, 2003) combined with low levels of IL-10 and are consequently strong promoters of Th1 immune responses. Alternatively activated macrophages or M2 demonstrate high production of IL-10 and low production of IL-12, as well as respond to IL-4 and/or IL-13 stimuli. Alternatively activated macrophages exert Th2-associated effector functions characterized by a high capacity for endocytotic clearance and antigen presentation accompanied by reduced pro-inflammatory cytokine secretion (Goerdt and Orfanos, 1999).

The involvement of IL-16 in immune regulation is complex. Its conventional function is described as a chemoattractant for human T cells by directly interacting with CD4. Another widely known function of IL-16 is to inhibit HIV-1 proliferation in infected CD4 + T lymphocytes. There are also some reports indicating IL-16 as a competent growth factor. Stimulation with IL-16 results in cell cycle progression in

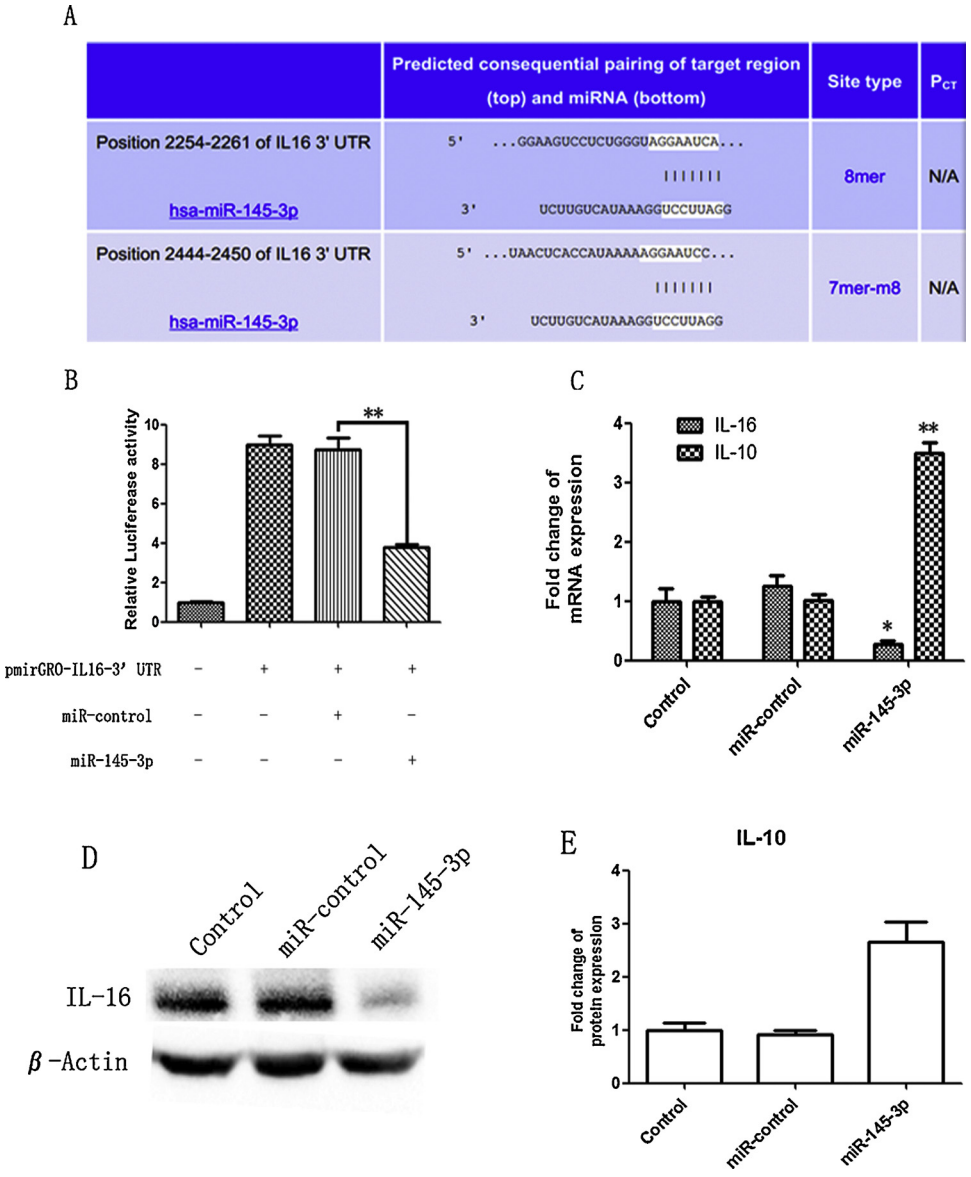

Fig. 4. Target prediction and verification of miR-145-3p on IL-16 and regulation of miR-145-3p in IL-16 and IL-10 in THP-1 cells. (A) The 3′UTR of IL-16 and miR-145-3p were aligned. The complementary sequences were marked. (B) Luciferase reporter plasmids containing the IL-16 3′-UTR were cotransfected into HEK293 T cells with miR-145-3p or control miRNA. 24 h after transfection, luciferase activity was measured. The normalized luciferase activity in the control group was set to 1. ** $P < 0.01$. (C) Quantitative PCR verification of IL-16 and IL-10 expression in M0 monocyte cells transfected with control miRNA or miR-145-3p mimic after 24 h. (D) IL-16 protein expression was detected by western blot. (E) IL-10 protein expression was detected by Elisa. n = 3.

CD4 + T lymphocytes (Blaschke et al., 1999; Parada et al., 1998). The role of IL-16 in monocytoid cell line differentiation and macrophage cell polarization after infection or during immune progression is nuclear, although it is expressed in many immune cells. Here, we reported that IL-16 expression is upregulated during M1 macrophage polarization, and IL-16 incubation enhances IL-6, IL-1α and IL-12 expression in macrophages, indicating M1 polarization. IL-16 also promotes the phagocytosis of macrophages. Knockdown of IL-16 with siRNA suppresses IL-16, IL-1α and IL-12 expression and decreases M0 cell apoptosis. It was proposed that CD4 action on target cells is required for mature IL-16 bioactivity. Elssner A et al. demonstrated IL-16 signaling through CD4 to activate the p38 MAPK and SAPK/JNK pathways in CD4+ macrophages (Elssner et al., 2004) Selective inhibition of JNK was shown to result in the greatest decrease in IL-6 and TNF-α expression (Baranova et al., 2008). These findings indicate that IL-16 may promote M1 polarization of macrophages through activating the p38 MAPK and SAPK/JNK pathways and enhancing IL-6 and TNF-α expression. Highly efficient phagocytosis is always characteristic of M2 microphages. However, in our results, IL-16 incubation enhanced the phagocytosis of M0 cells, though promoted M1 marker gene expression, indicating that immune cytokine secretion and phagocytosis are distinctly regulated by cell signaling. Mir-145 was reported to have a pro-M2 macrophage polarization function through promoting IL-10 expression in TLR4-triggered macrophages through directly targeting histone deacetylase 11 (Li et al., 2013; Shinohara et al., 2017). Through comparison, we found that miR-145 have two targets on the IL-16 3′UTR sequence, likely involved in IL-16 regulation during M2 macrophage polarization. We verified the target relationship of miR-145 on the IL-16 3′UTR by the dual luciferase reporter assay. M0 cells transfected with miR-145 mimic showed depressed expression of IL-16 and increased expression of IL-10. IL-16 knockdown with siRNA or miR-145 can promote IL-4- and IL-13-induced M2 polarization by enhancing CD206 and IL-10 expression but has no influence on CD163 expression. This may be due to CD 163 being highly induced by IL-4 and IL-13 incubation in the given time (72 h).

In conclusion, our results indicated that IL-16 is involved in macrophage polarization through regulating IL-6, IL-1α and IL-12 expression. IL-16 is a miR-145 target gene and is potentially responsible for miR-145-induced M2 polarization. The influence of IL-16 on macrophages needs to be further defined, and parallel experiments with additional cell lines are warranted in our further research. We observed no proliferation effect of IL-16 on THP-1 cells, but its knockdown benefitted M0 cell maintenance. Thus, the mechanism of IL-16 regulation of THP-1 and macrophage cell activity may be different from that in T

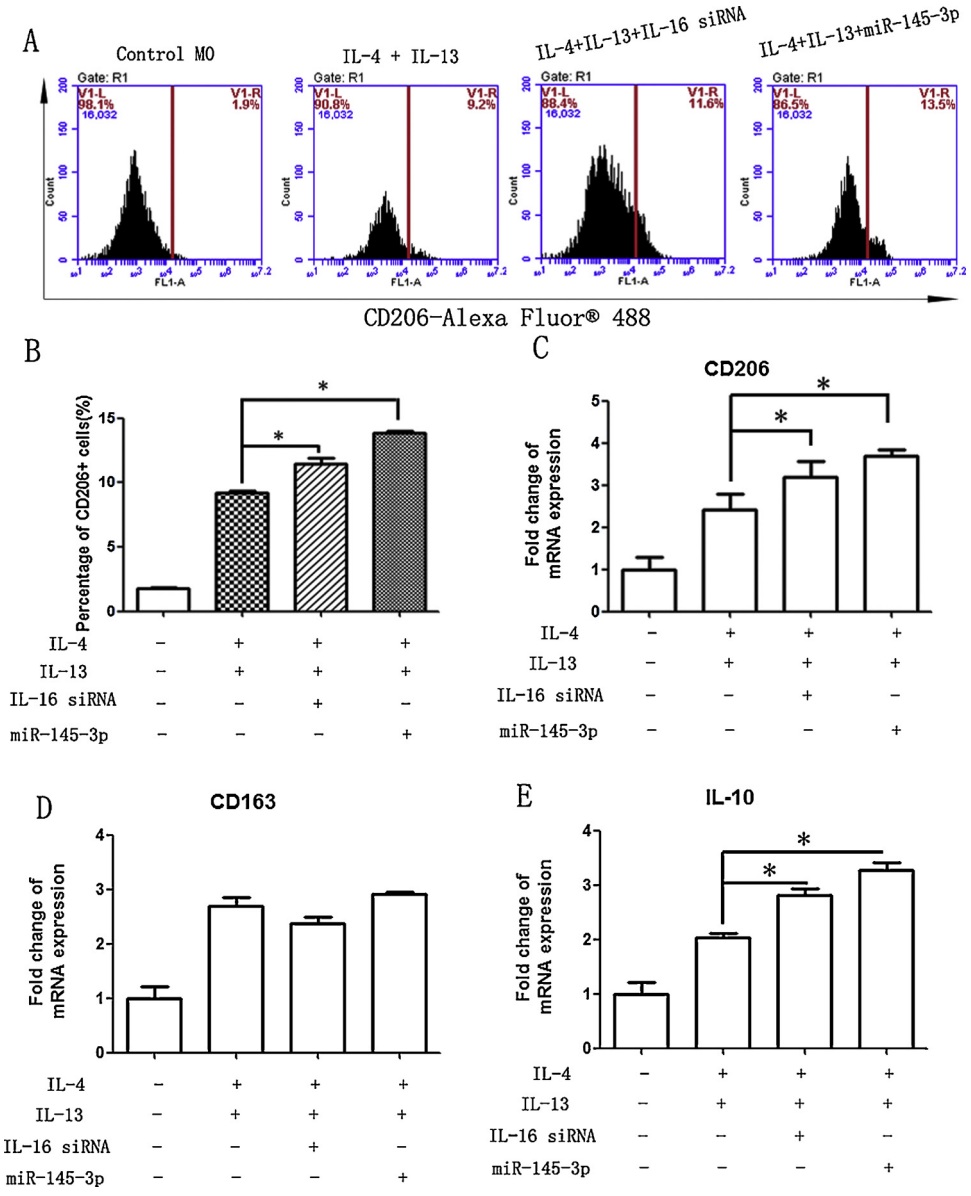

Fig. 5. Downregulation of IL-16 with mir-145 and IL-16 siRNA promotes M2 macrophage polarization. (A) FACS of the percentage of CD206+ cells under different M0 polarization conditions. (B) The percentage of CD206+ cells was presented as histone graphs. (C–E) Quantitative PCR verification of the expression of M2 macrophage marker genes CD 206, CD 163 and IL-10 under different inducing conditions.

lymphocytes.

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